TRANSPLANTATION OF ISLETS OF LANGERHANS

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J.R.Nash

The inadequacy of the present management of diabetes mellitus is reflected in the high morbidity and mortality caused by the long-term complications of the disease. Present evidence suggests that this is due to the inability to achieve strict diabetic control. This could possibly be attained by the successful transplantation of isolated islets of Langerhans. Rejection is the major cause of graft failure after transplantation. The susceptibility of isolated islet allografts to rejection was assessed in non-immunosuppressed and immunosuppressed rats, and their survival compared with that of other allografts.

Streptozotocin-induced diabetic rats received in excess of 500 islets from six adult donors. The pancreases were excised, digested with collagenase and after separation from the unwanted exocrine tissue, on a Ficoll gradient, the isolated islets were transplanted intraportally. Functional viability was confirmed by in vitro and in vivo tests.

The median survival of islet allografts in untreated recipients was significantly shorter than the median survival of kidney, heart and skin allografts transplanted across the same immunological barrier. Recipient immunosuppression with azathioprine, azathioprine and prednisolone, antilymphocyte serum or cyclophosphamide did not improve islet allograft survival. Macrophage suppression, using silica, resulted in the indefinite survival of seven out of sixteen allografts. Specific immunosuppression was more effective in prolonging islet allograft survival but not as effective as that for organ allografts. Passive enhancement resulted in the indefinite survival of four out of ten allografts. Graft survival was not improved by using serum from rats bearing a functioning, enhanced, islet allograft or by transplanting islets into rats bearing a functioning, enhanced, donor strain heart allograft.

It was concluded that isolated islet allografts are more susceptible to rejection than organ allografts and that present immunosuppression is less effective in abrogating their rejection. The present state of human islet transplantation was reviewed.

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INTRODUCTION

There is no doubt that the discovery of insulin, or isletin, as it was originally called, by Banting and Best in 1921 heralded a major breakthrough in the management of Diabetes Mellitus. Within months a disease which was inevitably fatal was transformed into one compatible with a good expectation of life. Their work was the major turning point in the history of diabetes, but significant contributions had been made by many other workers especially during the thirty years leading up to 1921.

The first known reference to diabetes is recorded in the Ebers papyrus, said to have been written about 1500 B.C. (Wrenshall et al 1962). The name diabetes was first used many years later by Aretaeus, a Cappadocian who lived around 200 A.D. Mellitus, the Latin for honey-sweet, was added to the name of the disease by Thomas Willis, physician to Charles II and a founder member of the Royal Society. It was a hundred years later that Matthew Dobson, a Yorkshireman, demonstrated the presence of sugar in the urine of diabetics. In these early years the progress of research in diabetes was slow, but many well documented descriptions are extant which show its true course and outcome, something not seen today other than in experimental animals.

Islets of Langerhans were discovered in 1869 but it was not until Von Mering and Minkowski (1889) demonstrated that total pancreatectomy in dogs resulted in severe diabetes that they were linked with the disease. The islet

theory was confirmed by Ssobolew and Schulze (Benseley 1915) who showed that in a duct ligated pancreas the acinar tissue degenerated fairly rapidly leaving viable islets, and that the animals used in these experiments did not develop diabetes. Opie (1901), approaching the problem from a different angle, reported that some patients dying of diabetes had shrunken and degenerate islets of Langerhans. During the early years of the twentieth century a great deal of work was done on the chemical nature of diabetes and many workers attempted to produce active extracts of the pancreas, but failed. It was against this background of repeated failure that Banting took up the challenge and, with Best's help, discovered insulin (Banting and Best 1922).

Over the years since 1921 much greater understanding of the action of insulin and the management of diabetes has been achieved. Long acting insulins, and more recently, oral hypoglycaemic agents have been introduced. There can be no doubt that the life expectancy of diabetics has greatly improved and that deaths due to diabetic coma have declined rapidly over the years but, as a result of their increased life-span, new long-term complications have come to light. These complications, which are predominantly degenerative changes involving the cardiovascular system, kidney and eye, are today responsible for the morbidity and mortality associated with diabetes.

The extent of the problem is difficult to assess, but it was estimated by the American National Commission on Diabetes (1975) that the life expectancy of diabetics is approximately 30% less than that of the general population.

Diabetics are, according to their findings, 25 times more prone to blindness, 17 times more prone to renal disease, 5 times more prone to gangrene and twice as prone to heart disease. This Commission also reported that the prevalence of the disease increased by 50% between the years 1965 and 1973. Many surveys have been conducted in order to assess the prevalence of diabetes, but individual surveys cannot be extrapolated to cover world populations in view of the wide geographical variations (Malins 1972).

A local diabetic survey, the Ibstock survey, (Walker & Kerridge 1961) of the inhabitants of a Leicestershire village undertaken in 1957 revealed a prevalence of 1.4%. Thus, twenty years ago a general practitioner with an average sized list of patients, approximately 2,500, had about 35 diabetics under his care. On a larger scale, the Leicestershire Area Health Authority, with a population of 836,500 in 1975, would have had a diabetic population of around 11,700. This figure does not take into account any change in prevalence over the last 20 years. Dr. Walker (1961) also showed, as others have done, that the incidence of diabetes increases with age, obesity, and a family history of the disease. It is thus not surprising that the prevalence of diabetes is rapidly increasing as reported by the American National Commission on Diabetes (1975), and today we would expect a much higher prevalence in Leicestershire than 20 years ago.

The management of the long-term complications in our rapidly expanding diabetic population presents many problems

and is far from adequate, so it would appear much more satisfactory to prevent their onset. This could be achieved either by preventing the onset of the disease if its aetiology were known, or possibly by maintaining the blood sugar under more physiological control. Transplantation of whole pancreas or isolated islets of Langerhans may achieve the latter.

In Chapter I, I will examine some of the evidence that the control of diabetes should be such that the blood sugar be kept as close to normal levels as is feasible in an attempt to prevent long-term complications. Although this might seem obvious it is by no means universally accepted (Ingelfinger 1977). This is of critical importance to the concept of managing diabetics by transplantation as its aim is the absolute normalisation of the blood sugar.

In Chapter II, I will review the methods of isolating and transplanting viable islets of Langerhans and describe in detail the technique which I adopted.

Early studies on the transplantation of rat islet allografts suggested that isolated islets were particularly susceptible to rejection (Reckard and Barker 1973). In Chapter III, I will present the results of islet allograft transplantation using our rat model and compare their survival with kidney, heart and skin allografts transplanted across the same immunological barrier.

The efficacy of both specific and non-specific immunosuppression in prolonging islet allograft survival in rats is presented in Chapter IV.

Finally, in Chapter V the present state of islet transplantation in man will be reviewed.

CHAPTER I

ISLET TRANSPLANTATION IN THE

MANAGEMENT OF DIABETES MELLITUS

- 1. INTRODUCTION
- 2. <u>THE RELATIONSHIP OF DIABETIC CONTROL TO</u> <u>THE DEVELOPMENT OF LONG-TERM COMPLICATIONS</u>
 - a) Animal Studies
 - b) Clinical Studies

3. <u>THE PATHOGENESIS OF DIABETES</u>

a) Islet Cells and Diabetogenic Hormones

<u>ي</u> .

- b) Types of Diabetes
- 4. CONCLUSION

CHAPTER I

ISLET TRANSPLANTATION IN THE MANAGEMENT OF DIABETES MELLITUS

1. INTRODUCTION

Whole pancreas and isolated islet transplantation have been undertaken in man (Chapter V) but are still experimental procedures. The justification for displacing insulin and oral hypoglycaemic agents in the management of diabetes must be clearly established before transplantation is offered as an alternative treatment. In this chapter I will discuss some of the evidence favouring the attainment of good diabetic control and the possible role of transplantation. I will also discuss the influence that the pathogenesis of diabetes may have on the function of transplanted islets.

2. <u>THE RELATIONSHIP OF DIABETIC CONTROL TO</u> THE DEVELOPMENT OF LONG-TERM COMPLICATIONS

A conclusive study demonstrating the efficacy of absolute normalisation of the blood sugar in diabetics is impossible to perform as there are, at present, no means of achieving such control. It is only possible to compare the results of different degrees of control and these studies are difficult to interpret. The ideal controlled prospective trial is not ethical as one cannot have a control group receiving less than the currently accepted treatment (Tunbridge 1975). The division into poorly and well controlled groups is achieved by assessing the patients' response to treatment, but this obviously introduces a bias against the less well controlled groups. Even the criteria for judging the degree of control are controversial (Lefebvre et al 1974; Ingelfinger 1978).

In reviewing the evidence in favour of a rigid control of diabetes I will discuss information obtained from animal studies and from clinical trials.

a) Animal Studies

Chemically induced diabetes in rats (Orskov et al 1965; Olsen et al 1966), dogs (Bloodworth et al 1969) and monkeys (Gibbs et al 1966) has resulted in pathological changes similar to those seen in man. Successful islet transplantation in diabetic rats results in regression or arrest of these changes (Mauer et at 1974; Gray and Watkins 1976). Similar beneficial effects are seen after whole organ transplantation (Weil et al 1974; Orloff et al 1975).

Fox et al (1977) compared glomerular basement membrane thickness in treated and untreated streptozotocin-induced diabetic rats with mean plasma glucose concentration and found a highly significant positive correlation. A further study of importance is that by Lee et al (1974) who showed that normal rat kidneys transplanted into diabetic rats developed morphological changes of diabetes whereas kidneys from diabetic rats transplanted into normal rats showed regression of the diabetic glomerular changes. They also reported that two diabetic patients who had received renal transplants, three and four years previously, showed diabetic vascular and glomerular lesions in the transplanted kidney. Engerman et al (1977) found that after five years, microvascular changes in the eyes of poorly controlled diabetic dogs were significantly worse than those in well controlled dogs.

It is by no means universally accepted that diabetic lesions in experimental animals are the same as those seen in man (Cameron et al 1975; Siperstein et al 1977). This would not be surprising as there is a marked species difference, but the observation that absolute normoglycaemia in these animals prevents or reverses the development of diabetic-like lesions is of great significance. There is no evidence to suggest that this cannot also be achieved in man.

b) <u>Clinical Studies</u>

In 1964 Knowles presented a review of 300 papers on the subject of diabetic control. These papers came from

85 centres, 50 of which considered that there was a positive correlation between vascular disease and poor control of diabetes, 25 thought that there was no relationship and 10 were undecided. Knowles (1964) felt that the information obtained was insufficient to reach any decisive conclusion. Nine years later Kaplan and Feinstein (1973), reporting on their evaluation of 149 papers, suggested that the widespread use of unsatisfactory methodology prevented satisfactory statistical analysis of therapeutic trials. The often quoted University Group Diabetes Program (UGDP 1976) reported no difference in the development of vascular complications between patients treated with insulin or tolbutamide, and an untreated group. However, this study was on maturity onset diabetics all of whom could have been treated by diet alone. There was also very little difference in blood sugar levels between the groups and none achieved strict control. As stated by Raskin (1978), there is little convincing clinical evidence favouring rigid control because few diabetics have ever been rigidly controlled. It is important to note that these reviews provide no evidence that vascular complications are independent of hyperglycaemia.

Recently the American Diabetic Association accepted as policy the view that better control is beneficial (Cahill et al 1976). In coming to this decision they took into account certain evidence: the results of animal studies, evidence that renal and muscle biopsies do not show abnormalities during the first few years of insulin dependent diabetes (Osterby 1972; Jackson et al 1975; Williamson and Kilo 1977), and the result of a recent prospective randomised study showing that more stringently controlled diabetics develop less severe retinopathy (Job et al 1975). A number of other reviews also support the need for good diabetic control (Stowers 1975; Tchobroutsky 1978). A field of particular interest is the management of diabetes in pregnancy. A review by Watkins (1978) concluded that progressively more obsessional control of diabetes during pregnancy and labour had been accompanied by a considerable improvement in foetal survival.

Siperstein et al (1977), who are not convinced that strict diabetic control will prevent long-term complications, make two significant statements: firstly, that it is wise to strive for reasonable control and secondly, that at no time have they suggested that good control might not have value. Their reason for suggesting caution is to avoid hypoglycaemic episodes in patients striving too hard to achieve normoglycaemia because of the harmful effects that these episodes may have (Bloom et al 1969: Bale 1973).

Clinical studies have so far failed to achieve uniform acceptance of the value of strict diabetic control. This failure would appear to reflect the present inability to achieve such control. Attempts are being made, using continuous insulin infusion, to achieve absolute normoglycaemia (Genuth and Martin 1977; Pickup et al 1978) but these techniques have only been used in shortterm studies and have not been fully evaluated.

3. THE PATHOGENESIS OF DIABETES

a) Islet Cells and Diabetogenic Hormones

Islets of Langerhans contain four different cell types: alpha cells which secrete glucagon, beta cells which secrete insulin, delta cells which secrete somatostatin and delta one cells which secrete pancreatic polypeptide. The interrelationship between these hormones is complex and poorly understood (Bloom 1978). Unger (1971) suggested that the ratio of insulin to glucagon was important in maintaining normal caloric homeostasis. He took this further in the Banting Memorial Lecture in 1975 (Unger 1976) when he postulated a "double trouble" theory to explain the raised blood sugar in diabetes. This theory proposed that hyperglycaemia was not only due to the underutilisation of glucose as a result of absolute or relative insulin deficiency, but also to the over-production of glucose due to an absolute or relative excess of However, Barnes et al (1975) suggested that glucagon. glucagon was unlikely to play a major role in the control of blood sugar levels in insulin-dependent diabetics.

Somatostatin has an inhibitory effect on the production of growth hormone, insulin, glucagon and many other hormones (Lancet 1975). At one stage it was hoped that it would be useful in the treatment of long-term diabetes but its actions seem too widespread for it to fulfil an important therapeutic role (Lancet 1975). The action of pancreatic polypeptide in man is unknown, but Floyd and Fajans (1976) have shown that it is significantly elevated in both juvenile and maturity onset diabetes. Gepts et al (1977) have recently reported hyperplasia of delta one cells in the pancreas of juvenile diabetics.

Pancreatic islet cell hormones are not the only hormones which affect glucose metabolism. Insulin secretion is considerably greater when glucose is given orally than when it is given intravenously, which suggests that 'gastrointestinal hormones' released in response to food stimulate beta cells (McIntyre 1964). Growth hormone, cortisol, adrenaline, thyroid hormones and oestrogens also influence glucose metabolism such that abnormalities in their production may produce hyperglycaemia. Growth hormone is of particular interest as it not only has a diabetogenic effect but it has also been directly implicated in the actiology of diabetic angiopathy. Growth hormone levels are elevated in newly diagnosed diabetics and in those of some years duration (Johansen and Hansen 1971), but after a period of strict metabolic control growth hormone secretion is normal (Hansen 1971). The results are difficult to interpret but it appears that whenever there is a combination of hyperglycaemia and elevated serum growth hormone microangiopathy is found (Albertiand Hockaday 1975).

The incomplete understanding of the relative roles of these hormones in normal subjects and in diabetics makes it difficult to postulate the influence of islet transplantation. The progressive disappearance of beta cells from the islets of juvenile-onset diabetics

and the preservation and even hyperplasia of alpha, delta and delta one cells (Gepts et al 1977) upsets the balance which insulin administration is unable to fully correct. The transplantation of islets would establish an islet cell mass with normal beta cells. This would, hopefully, by normal homeostatic means correct the hormonal imbalance and re-establish normal glucose metabolism.

b) Types of Diabetes

Diabetics can be loosely grouped into two main types of diabetes: juvenile-onset diabetes (Type I) and maturityonset diabetes (Type II). There are a number of other syndromes in which a disordered glucose metabolism plays a part, but as they are uncommon and form a very heterogeneous group they will not be discussed further.

i) Juvenile-onset Diabetes

This type of diabetes is characterised by a rapid onset with symptoms such as polydipsia, polyuria, loss of weight and lassitude. It usually presents in childhood or early adult life and treatment with insulin is necessary.

The influence of heredity is unclear and variable. Bloom et al (1975) reported a history of diabetes in first-degree relatives in 11% of patients whereas Calman and Peckham (1977) found such a history in 23% of juvenileonset diabetics. The incidence of first-degree relatives with diabetes in non-diabetic children is about 1% (Calman and Peckham 1977). These figures suggest that there must be a genetic factor although not a strong one. Of particular interest is the finding by Cudworth and Woodrow .(1975) that the frequency of the histocompatibility antigens HLA-A8 and W15 is increased in juvenile-onset diabetics. For a number of years viruses have been implicated in the aetiology of diabetes (Steinke and Taylor 1974) and evidence presented by Cudworth et al (1977) suggested that the possession of these HLA haplotypes may confer a susceptibility to certain virus infections. Lendrum et al (1976) and Irvine et al (1977), in two large series, have established the presence of antibodies to islet cells in juvenile-onset diabetics. In the study presented by Lendrum et al (1976) the prevalence in those tested within a week of onset of symptoms was 86%. This declined to between 10% and 20% by two to three years.

Bloom (1978) postulated that present evidence suggests that HLA phenotypes confer a susceptibility to virus infections pathogenic to islet cells, and that damaged islet cells release antigenic products which lead to the production of antibodies. The antigen-antibody reaction then destroys the beta cells giving rise to diabetes.

Transplantation would probably be of most benefit to juvenile-onset diabetics as they are insulin-dependent and have the disease for most of their lives. If Bloom's (1978) explanation of the aetiology is correct the islet cell antibodies are unlikely to destroy the transplanted islets as they will have different antigenic determinants, and although the patient will still possess the susceptible HLA phenotype, immunity to the virus should have developed after the initial exposure.

ii) Maturity-onset Diabetes

As its name implies this type tends to develop in middle-age. The symptoms are mild and often insidious, presenting with diabetic complications rather than an acute illness. For example, deterioration of sight as a result of diabetic retinopathy or foot ulceration as a result of neuropathy and angiopathy. This type is distinct from juvenile-onset diabetes in that there is a normal or high insulin output. The blood sugar responds to a reduced intake of carbohydrates either alone or in conjunction with oral hypoglycaemic agents and treatment with insulin is seldom necessary. However, these patients still develop diabetic complications.

Maturity-onset diabetes, which is not HLA linked has a strong genetic background (British Medical Journal 1975). It has been estimated that 60% of the children of two maturity-onset diabetics will develop mild diabetes by the age of 60 (Tattersall and Fajans 1975). Cesai and Luft (1963) demonstrated a sluggish insulin response to sustained hyperglycaemia which would explain why the beta cell defect becomes evident at a time of environmental or hormonal stress. Archer et al (1975) showed that cells from obese patients had a reduced affinity for insulin as a result of a decreased insulin receptor concentration. It is thus not surprising that a combination of insulin insensitivity in obese patients and genetically imperfect beta cells produces diabetes. They went further to show that if obese patients were treated with a strict low calorie diet the number of receptor sites increased.

The development of long-term complications in patients with maturity-onset diabetes demonstrates the inadequacy of present management. Normoglycaemia achieved by islet transplantation may prevent these complications although the age of the patient may be a contraindication to such treatment.

4. CONCLUSION

The aetiology of diabetes is still not completely understood and only when it is will we know the influence it might have on transplantation. At present there is no evidence to suggest that transplanted islets would be rejected in a manner other than would be expected from a normal immune response.

Clinical studies investigating the value of strict diabetic control are inconclusive although the overall weight of opinion favours such control. Successful transplantation as achieved in animals may prove the only method of resolving the controversy.

CHAPTER II

ISLET ISOLATION AND TRANSPLANTATION

1. <u>INTRODUCTION</u>

2. <u>MATERIALS AND METHODS</u>

- a) Animals
- b) Induction of Diabetes
- c) Isolation of Islets of Langerhans
- d) Transplantation of Islets

3. ASSESSMENT OF ISLET VIABILITY

- a) In Vitro Test of Functional Viability
- b) In Vivo Test of Functional Viability
- c) Conclusion

CHAPTER II

ISLET ISOLATION AND TRANSPLANTATION

1. INTRODUCTION

Moskalewski (1965) was the first to describe the isolation of islets of Langerhans from the acinar portion of the pancreas by enzymic digestion. He excised the pancreas from the rat, diced it into small pieces, and after thorough washing digested it in a collagenase solution. The enzymic digestion was terminated at the stage when the islets were seen lying free from the exocrine tissue. The digested tissue was washed and allowed to sediment out for one minute, the supernatant being discarded. The precipitate was dispersed in Hanks' solution and the islets were identified under a dissecting microscope and picked out with a cataract He was able to culture the islets and undertake knife. studies on the effect of varying glucose concentrations on their morphology and function.

A few years later Lacy and Kostianovsky (1967) described their technique of islet isolation. It was based on the collagenase digestion technique of Moskalewski (1965), but with two modifications. Firstly, to aid digestion the pancreas was distended and disrupted by injecting Hanks' solution into the distally ligated common bile duct before excision of the pancreas. Secondly, to improve the yield of islets obtained from the digested tissue, they layered the tissue onto a discontinuous sucrose gradient, using 8 - 10 ml layers of 1.4, 1.6, 1.7 and 1.8 molar solutions. After centrifuging for 15 minutes at 2,600 rpm the islets were lying free on the upper surfaces of the 1.6 and 1.7 molar layers. Approximately 200 to 300 islets were obtained from a single rat pancreas. Although this technique increased the yield of isolated islets, subsequent studies with these islets suggested that they had been damaged, probably as a result of contact with the hyperosmolar sucrose solutions (Lacy and Kostianovsky 1967).

Two years later Lindall et al (1969) reported the use of a Ficoll density gradient to separate out the islets, thus avoiding their contact with hypertonic sucrose solutions. Ficoll is a synthetic polymer made by the copolymerization of sucrose and epichlorohydrin. Its high molecular weight, high density, low viscosity, low osmotic pressure and non-ionised state, render it an ideal solute for a density gradient (Pharmacia Fine Chemicals).

Using the Ficoll gradient technique Ballinger and Lacy (1972) reported the isolation of approximately 200 islets per rat pancreas and the subsequent intraperitoneal transplantation of 400-600 isolated islets into diabetic recipient rats. Although the islet isografts reduced the overall mortality of the recipient rats as compared with a control group there was a wide variation in response as shown by the recordings of blood sugar and urine volume. Scharp et al (1973) discussing the initial work by the St. Louis group suggested that the variable results of islet transplantation could be explained by the damaging effect of impurities in commercially available Ficoll. Wallach

and Kamat (1964) had previously reported the contamination of Ficoll with small quantities of sodium chloride and sucrose polymers of low molecular weight. They dialysed the Ficoll against deionised water before use. Scharp et al (1973) demonstrated, with in vitro continuous perifusion studies on insulin release from islets, the difference between dialysed and non-dialysed Ficoll, and concluded that preliminary dialysis of Ficoll was indicated.

In setting up a technique for islet isolation I undertook studies on the viability of islets separated on an undialysed Ficoll gradient as the added time and expense of dialysis and subsequent lyophilisation was not inconsiderable. I found that it was unnecessary to dialyse the Ficoll (Nash et al 1976). This will be discussed in greater detail later in this chapter.

Shortly after Ballinger and Lacy published their work on the isolation and transplantation of islets, a group from Minnesota (Lennard et al 1973) reported a different approach to the problem of harvesting a sufficient number of viable islets for transplantation. They recommended a similar collagenase digestion technique, but instead of the adult rat pancreas they used 20-35 neonatal rat pancreases between $2\frac{1}{2}$ and $4\frac{1}{2}$ days postpartum. At this age the rat pancreas has the highest insulin concentration, the largest islet cell volume (Hegre et al 1973) and the lowest exocrine enzyme concentration (McEvoy et al 1973) than at any other stage in neonatal or adult life. Using this method it is possible to transplant the digested and washed pancreatic tissue directly into the peritoneal cavity

without isolating the islets, as the poorly developed exocrine tissue does not produce peritonitis. Islet wastage is thus reduced to a minimum. This technique seemed ideal for my purpose but a pilot study done at the outset highlighted two problems. Firstly, the large number of neonatal rats required for the transplants put great pressure on our source of supply. Secondly, there was a prolonged delay in the recipient returning to normoglycaemia after intraperitoneal transplantation. This delay, as shown by Lennard et al (1973) was such that in the allograft experiments rejection occurred before normoglycaemia was established.

The work of Kemp et al (1973a,b) is of particular interest in regard to this latter point. They demonstrated that the site of transplantation was critical for rapid lowering of the blood sugar. Intrahepatic dispersal of isolated islets injected via the portal vein proved an ideal site, resulting in both a rapid lowering of the blood sugar and a more effective control of diabetes. Recently other workers have reported the intra-splenic site as an effective site for transplantation (Koncz et al 1976) (Finch et al 1977). The spleen has the advantage of being easily removed and thus the survival of transplanted islets can be confirmed. Using this method it is also possible to transplant digested and washed adult pancreas without the need for prior isolation of the islets and resultant exclusion of exocrine tissue (Mirkovitch and Campiche 1976) (Kretschmer et al 1977). The critical factor relating to transplant site was shown

by Mullen et al (1977) when they demonstrated the need to deliver the insulin directly into the portal circulation. After transplanting neonatal pancreases under the kidney capsule control of diabetes was greatly enhanced by constructing a shunt between the renal vein and portal vein.

In order to study the rejection of isolated islets, I required a method of islet transplantation which resulted in the rapid establishment of normoglycaemia in the diabetic recipient. For this reason I adopted the technique of isolating adult rat islets (Ballinger and Lacy 1972) with subsequent intra-portal transplantation (Kemp et al 1973a,b).

2. MATERIALS AND METHODS

a) Animals

Inbred adult rats of either sex were used throughout the study. Inbred Wistars were used for the isograft experiments. Swiss albino (AS) rats acted as recipients for the allograft experiments and (AS x August) F_1 hybrid rats as donors (Figure 1). A different strain was used for the isograft experiments because of limited supplies of inbred AS rats. The rats had free access to food and water.

b) Induction of Diabetes

The recipient rats were made diabetic at least two weeks before transplantation by an intravenous injection of freshly prepared streptozotocin (Upjohn Co., Kalamazoo, Michigan). Streptozotocin is an antibiotic extracted from Streptomyces acromogenes (Herr et al 1959) which is diabetogenic in rats when given intravenously (Rakieten et al 1963). Junod et al (1967) have shown that it has a specific, rapid and irreversible cytoxic action on pancreatic beta cells.

At the outset a dose of 60 mg/kg of streptozotocin was used but due to the high morbidity and mortality of the rats this was reduced to 50mg/kg. A streptozotocin dose-response curve (Ganda et al 1976) showed it to have no significant effect at a dose of less than 20mg/kg but to be highly effective at a dose of greater than 40mg/kg. Before injection the streptozotocin was dissolved in 1ml of normal saline which had been adjusted to a pH of 4.2 with 0.05 M citric acid. This is necessary as it is inactivated at a neutral pH (Rakieten et al 1963). During the course of my experiments a few rats failed to respond to streptozotocin injection, but these could easily be identified in the interval before transplantation. The instability of the agent could explain the failure in these rats. Rats confirmed to be diabetic after streptozotocin injection did not spontaneously revert to normoglycaemia whilst under observation.

c) Isolation of Islets of Langerhans

Six young donor rats weighing between 150 and 250 gms were used for each transplant. Under ether anaesthesia the common bile duct was displayed through a midline abdominal incision (Figure 2). The distal end of the duct was occluded as it entered the duodenum with a small pair of artery forceps; the proximal end was cannulated with a 23 gauge butterfly needle which was held in place by a cotton ligature (Figure 3). The pancreas was distended and disrupted by the slow injection of 10mls of cold Hanks' solution into the common bile duct (Figure 4). The pancreas was then totally excised and the rat killed.

The pancreases from the six rats were pooled, washed with cold Hanks' balanced salt solution and diced into small pieces (Figure 5). Once the fragments were about 1-2mm in diameter (Figure 6) the tissue was washed again and the supernatant discarded. The tissue was then digested with collagenase to separate the islets from the unwanted exocrine tissue. A mixture of Boehringer and Sigma type II
collagenase was used. The quantities altered slightly with different batches of the enzymes, but were usually 7.5 mg Boehringer and 2.5 mg Sigma per pancreas. Sufficient enzyme to digest two pancreases was placed in each of three 50 ml siliconised conical flasks. The diced pancreatic tissue was divided between the three flasks and the volume made up to approximately 5 mls with Hanks' solution. Digestion was then undertaken in a water bath at 37°C, the flasks being vigorously agitated by hand or using a flask shaker. The end point of digestion was determined by assessing the degree of islet separation under a dissecting microscope. Once the islets were seen to be free from the exocrine tissue, digestion was stopped by adding cold Hanks' solution, the procedure taking 5-10 minutes.

The digested tissue was washed three times in 50 ml siliconised tubes. After each washing the tubes were allowed to stand for three minutes before the supernatant was removed. The residue was transferred to 15 ml siliconised conical test tubes and centrifuged. The supernatant was discarded.

The islets were separated from the unwanted exocrine tissue on a discontinuous Ficoll gradient (Figure 7). The pellet of pancreatic tissue was dispersed in 4 mls of 25% Ficoll (W/V made up in Hanks' solution); onto this 2 ml quantities of 23%, 20% and 11% Ficoll were carefully pipetted in layers of decreasing concentration. The three tubes were centrifuged for 10 minutes at 800g. The islets were then removed from the 20% layer and washed in

Hanks' solution. More than 500 islets were used for each transplant.

d) <u>Transplantation of Islets</u>

Under ether anaesthesia and through a midline incision the portal vein was displayed. The islets, dispersed in 0.5 - 1ml of Hanks' solution, were drawn into a 1ml siliconised glass syringe and injected into the portal vein through a 25 gauge needle (Figure 8). To ensure that all the islets had been injected blood was swilled in and out of the syringe before removing the needle. Haemostasis was secured with a small piece of absorbable gelatin sponge (Allen and Hanburys Ltd., England) and gentle pressure.

After transplantation the recipients were bled daily from the tail vein for estimation of their blood sugar (Appendix A) for the first two weeks, and then fortnightly up to the 100th day. Rejection was diagnosed if the blood sugar rose above 9 mmol/Litre.







Fig. 2 Occlusion of the distal end of the common bile duct and eversion of the liver onto the chest wall displaying the proximal end of the duct.



Fig. 3

Cannulation of the common bile duct; the needle held in place with a ligature.



Slow injection of cold Hanks' solution into the common bile duct, distending and disrupting the pancreas. Fig. 4



Fig. 5 Manual dicing of the six pancreases.



Fig. 6

The pancreatic tissue is diced until the fragments are 1 - 2mm in diameter.



Fig. 7 Ficol

Ficoll gradient.



Fig. 8 Transplantation of islets from the six donors into the portal vein of the recipient.

3. ASSESSMENT OF ISLET VIABILITY

Once the technique of isolating a sufficient number of islets had been established their functional ability was assessed. This was particularly important as Scharp et al (1973) had reported the toxicity of commercially available Ficoll. They had found that islets separated on a Ficoll gradient had a severely impaired response to glucose stimulation.

An in vitro test of viability was carried out by measuring the insulin release in response to glucose stimulation of islets separated by a manual technique as compared with those separated on a Ficoll gradient (Nash et al 1976). In vivo viability was assessed by transplanting islets between members of the same inbred strain, thus avoiding islet destruction due to rejection.

a) <u>In Vitro Test of Functional Viability</u>

Individual islets were harvested in two different ways: by a manual technique which involved pouring part of the digest into a Petri dish and picking out the islets under a dissecting microscope, and by the Ficoll gradient technique as described earlier in this chapter.

Groups of three islets were incubated for one hour in 1 ml of Krebs-Henseleit bicarbonate buffer containing 2g/L of bovine albumin (Armour Pharmaceutical, Eastbourne, England), and either 1.67 mmol/l or 16.7 mmol/L of glucose. Each test was performed in triplicate. After an hour 0.1 ml of the incubating fluid was withdrawn, diluted 5 times in buffer, and stored at -20° C to await insulin assay. The insulin was assayed using a double antibody radioimmunoassay (Appendix B).

The results of seven experiments, shown as a histogram in Figure 9, clearly demonstrate the ability of islets to respond to glucose stimulation: there was no statistical difference in response between those separated by the Ficoll gradient or manual techniques. From these results we concluded that there was no need to dialyse the Ficoll, contrary to the recommendations of Scharp et al (1973). The difference between our results could be explained by recent improvements in the production of Ficoll (personal communication from Pharmacia 1976).

b) In Vivo Test of Functional Viability

Successful transplantation of islet isografts, with long term amelioration of induced diabetes, has been reported by a number of groups (Ballinger and Lacy 1972: Leonard et al 1973; Ziegler et al 1974). From these studies it was clear that, provided the problem of rejection was excluded, normalisation of the blood sugar could be expected if the transplanted islets were viable.

Ten diabetic inbred Wistar rats were transplanted with islets from members of the same inbred strain. All but one became normoglycaemic (Table I). The recipient whose graft completely failed to function received an adequate number of islets, but the time taken in preparation and transplantation was $3\frac{1}{4}$ hours, which was $1\frac{1}{4}$ hours longer than normally taken. The work of Tellez-Yudilevich et al (1977) demonstrated the susceptibility of islets to warm and cold ischaemia.



Fig. 9 Insulin release in one hour incubations of three islets. The vertical columns show the mean of seven experiments: there is no significant difference between the response of manually or Ficoll separated islets at the two glucose concentrations.

Experiment No.	No. of days before B.S. <9 mmol/L	Graft survival (Days)
74		> 100
75	3	> 100
76	13	> 100
77	T T	8≠
79	1	>100
83	21	> 100
97	2	> 100
9 9	Never	+
102	1	20
104	8	> 100

* Died Normoglycaemic

+ Died Hyperglycaemic

Islets obtained from a rat pancreas maintained at room temperature for two hours had a normal response to glucose stimulation, but this was not the case if they were similarly preserved for four hours. After preservation at 4°C the basal insulin and the glucose stimulated insulin secretion remained normal for up to six hours after excision of the pancreas. It would thus appear that islets transplanted after $3\frac{1}{4}$ hours should still be viable. However, in our experience we found it difficult to maintain a constant temperature of 4°C, so in order to ensure islet cell viability we kept the time for islet transplantaion and isolation to less than two hours. Scharp (personal communication) suggested that the islets should be transplanted within one hour, but we could not achieve that speed and our results indicated that this was not essential.

None of the successfully transplanted recipients reverted to the diabetic state and four survived longer than one year after transplantation. The effect of transplanting a low yield of islets, less than 500, is shown in the results of experiments 76 (Figure 10) and 83. Matas et al (1976a) reported that it was possible to successfully transplant small numbers of islets. They demonstrated that the interval before normoglycaemia was achieved was inversely proportional to the number of islets transplanted.

Recipients receiving more than 500 islets rapidly reverted to normoglycaemia (Figure 11), although it was noted that this was not always achieved within 24 hours

of transplantation. This will be discussed further as it is of importance with respect to the allograft experiments. Experiment 104 is unusual in that the islets were diverted to the right half of the liver. This was an unsuccessful attempt to assist the location of individual islets for histological examination. This procedure may explain the extra delay in attaining normoglycaemia.

c) Conclusion

These studies demonstrated the viability of islets isolated by the method which I established in Leicester and enabled the allograft studies to be pursued in the knowledge that failure of graft survival was not due to damage of the islets of Langerhans during their preparation or transplantation.



Fig. 10 Transplantation of an insufficient number of islets resulting in a delayed return to normoglycaemia (Experiment No. 76).

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<u>Fig. 11</u> Transplantation of more than 500 islets resulting in a rapid return to normoglycaemia (Experiment No. 79).

CHAPTER III

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THE REJECTION OF ISOLATED ISLET ALLOGRAFTS

1. <u>INTRODUCTION</u>

- 2. <u>STUDIES ON THE SUSCEPTIBILITY OF</u> ISLET ALLOGRAFTS TO REJECTION
- 3. <u>DISCUSSION</u>

CHAPTER III

THE REJECTION OF ISOLATED ISLET ALLOGRAFTS

1. INTRODUCTION

Rejection remains the major cause of graft failure after cadaver renal transplantation. The one year graft survival is just over 50% (Figure 12), and this figure has not improved significantly over the last ten years (Human Renal Transplant Registry 1977). Some endocrine tissues, however, have been shown to be less susceptible to rejection (Russell and Gittes 1959) and it was originally hoped that isolated islet allografts would share this propensity. In support of this, Lillehei et al (1970) found that histological examination of rejected whole pancreas grafts showed little evidence of damage to the islets of Langerhans.

Reckard et al (Reckard and Barker 1973; Reckard et al 1973; Ziegler et al 1974) studied the survival of isolated islets transplanted, intraperitoneally, across differing histocompatability barriers. They found that islets transplanted from ACI (Ag-B4) to DA (Ag-B4) rats had a median survival of 8.4 days, whereas islets transplanted from Fischer (Ag-B1) to AC1 (Ag-B4) rats had a median survival of only 1.7 days. Compared with the survival of skin and heart allografts transplanted across the same histocompatibility barriers this represented very rapid rejection, and was the first evidence to suggest that isolated islets, far from being immunologically privileged,



Fig. 12 Survival of first cadaver renal grafts. (U.K. transplant: Annual Report 1976-77).

were more susceptible to rejection than other grafts.

Marquet and Heystek (1975) transplanted islets, intraportally, from Wag/Rij rats to AG-B incompatible Sprague-Dawley rats. In the untreated recipients graft survival ranged from seven to 12 days. However, they also reported that in the Wag/Rij to Sprague-Dawley donor-host combination skin grafts and heterotopic heart grafts were invariably rejected within 10 days. These results suggested that islets were no more susceptible to rejection than other grafts, in contradiction to the findings of Reckard and Barker (1973). The difference may have been due to the site of transplantation. Reckard and Barker used the intraperitoneal site whereas Marquet and Heystek dispersed the islets intrahepatically via the portal vein. This would suggest that the intrahepatic site was privileged.

In 1976 Finch and Morris reported that isolated islet allografts transplanted intraportally between Lewis (Ag-B1) and DA(Ag-B4) rats and from the (Lewis x DA) F₁ hybrid to the parent strains were rejected within two to six days after transplantation. These results provided further evidence of the susceptibility of isolated islet allografts to rejection. Our study (Nash et al 1977) (to be discussed in more detail in the next section) supported the findings of Reckard and Barker (1973), demonstrating that islet allografts.

2. <u>THE SUSCEPTIBILITY OF ISLET ALLOGRAFTS</u> TO REJECTION

While establishing our islet transplant technique we transplanted islets between members of our inbred Wistar colony to help confirm the viability of the isolated islets. The results of these experiments are shown in Table II. A11 the recipients reverted to normoglycaemia after transplantation, but within 14 days had become hyperglycaemic. The explanation for the graft failure was puzzling, but could only have been due to physical or chemical injury sustained during islet isolation, or due to islet rejection. The in vitro studies undertaken on the islets (Chapter II) confirmed that they were viable, so the isograft studies were repeated using rats from a different inbred Wistar colony. These grafts had an indefinite survival (Table I) demonstrating that islet destruction in the original experiment had been due to an immunological phenomenon rather than a manifestation of poor isolation and transplantation technique. We did not know the origin of the failure of inbreeding in our Wistar colony so were uncertain about the histocompatibility differences between donor and recipient, but assume it was very minor as heart grafts were readily accepted. The colony was in fact being used for all isograft work until the results of islet transplantation revealed the minor immunological barrier.

The results of islet transplantation across a major histocompatibility barrier are shown in Table III. (AS x Aug)F₁ hybrid rats acted as donors and diabetic AS rats as recipients. Graft survival in the ten recipients ranged

Treatment	<u>No. of rats</u>	No. of days before rejection
None	7	2, 2, 2, 5, 5, 6
		14

<u>Table II</u> Wistar. —> Wistar. The results of transplantation between members of our "inbred" rat colony.

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<u>Table III</u> (AS x Aug) $F_1 \longrightarrow$ AS. The survival of islet allografts in untreated recipients.

from zero to five days with a median survival of three days. In three recipients the blood sugar did not fall below 9 mmol/L. Despite the fact that sufficient islets had been transplanted, these three experiments were initially regarded as failures as it was impossible to be certain that damage to the islets had not occurred before transplantation. Reviewing these results it appeared that the failure to achieve normoglycaemia could have been due to rejection occurring within 24 hours as has been suggested by other workers (Vialettes et al 1978). On the other hand there could have been a similar delay in achieving normoglycaemia as seen in some of the isograft experiments (Table I). Under the latter circumstances it seems very likely that rejection would have intervened before normoglycaemia was achieved. An indication of graft function was obtained in one of these recipients as the blood sugar temporarily fell below pre-transplant levels (Figure 13). I have included these results even though the interpretation of them is open to some debate. However, it would seem that rejection before the establishment of normoglycaemia was the most likely cause of graft failure in these three recipients.

Islet allograft survival has been compared with the survival of kidney, heart and skin allografts transplanted across the same immunological barrier (Tilney and Bell 1974; Nash et al 1977). In these experiments heterotopic heart and kidney grafts were transplanted using standard microvascular techniques (Lee 1967; Ono and Lindşey 1969). The aorta of the cardiac graft was anastomosed end to side to the abdominal aorta of the recipient, and the pulmonary

artery was anastomosed in a similar fashion to the inferior vena cava. The kidneys were removed with a cuff of aorta and vena cava and transplanted into bilaterally nephrectomised recipients. The ureter was taken from the donor with a small patch of bladder which was sutured into a hole made in the dome of the recipient bladder. Skin grafts taken from the ears of the donor were sutured onto the flank of the recipient and a protective dressing placed around the animal for seven days after grafting.

Rejection of islet allografts was diagnosed if the blood sugar rose above 9 mmol/L (Chapter II). The status of heart grafts was assessed by palpating the ventricular contractions through the flank. Total cessation of the heart beat was regarded as evidence of rejection. The end point of rejection of renal grafts was taken as recipient death. Complete escharification of skin grafts was regarded as the end point of their rejection.

The kidney, heart and skin allografts had a median survival of 8.8 days ranging from seven to ten days (Table IV). Their survival was significantly longer than the median survival of islet allografts which was only three days. Islet allograft rejection was even more rapid than second-set rejection of heart allografts (Tilney and Bell 1974).



Fig. 13 Graph showing a transient fall in blood sugar after transplantation suggestive of initial graft function.

Tissue	No. of rats	No. of days before rejection	<u>Median time of</u> rejection
Islets	10	0, 0, 0, 2, 2, 3, 3, 3, 5, 5	3.0
Kidney	5	7, 9, 9, 9, 10	9.5
Heart	7	7, 7, 8, 8, 8, 8, 9	8.4
Skin	5	7, 8, 8, 9, 9	8.8

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<u>Table IV</u> $(AS \times Aug)F_1 \longrightarrow AS.$ Comparative survival of islet, kidney, heart and skin allografts in untreated recipients.

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3. DISCUSSION

The increased survival of the kidney, heart and skin allografts could in part be explained by the differing criteria used in assessing rejection. For example, if a rising blood urea was regarded as evidence of renal allograft rejection, rather than recipient death, the rejection times would have been very similar to the two islet grafts that were rejected on the fifth day. Even taking this into account it appeared that isolated islet allografts were more susceptible to rejection than vascularised organ grafts or skin grafts.

The time of rejection of the longest surviving islet allograft (Figure 14) corresponds very closely with the increase in cell-mediated immunity as measured by the Cr release assay (Figure 15) (Biesecker 1973; Burgos et al 1974). This finding is in keeping with the concept that allograft rejection is mediated by sensitised lymphoid cells (Mitchison 1955). Using a different test of cellmediated immunity, the microcytotoxicity assay, Biesecker et al (1973) have detected significant cell-mediated immunity three days after renal transplantation (Figure 16). Biesecker et al (1973) postulated that the difference between the Cr release assay and the microcytotoxicity assay may lie in the measurement of different cell populations or reflect a difference in immunological response of the effector cell population to varying in vitro conditions in the two techniques. The rapidity of islet allograft rejection may reflect a particular sensitivity to cellmediated immunity, the variability being attributable to





Fig. 15 The ⁵¹Cr release assay: cellular immune response of spleen cells measured at varying times after renal transplantation in the rat. (Biesecker 1973)



Fig. 16 The microcytotoxicity assay: cellular immune response of spleen cells measured at varying times after renal transplantation in the rat. (Biesecker et al 1973).



<u>Fig. 17</u> (AS x Aug) $F_1 \longrightarrow$ AS. Rapid rejection of an islet allograft.

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differing immunological activities of the effector cell population.

There is very little evidence, however, to implicate cell-mediated immunity in the failure of grafts which were rejected at a very early stage (Days 0-3) (Figure 17). At this stage the 51 Cr release assay was negative and cellular immunity was only weakly detected by the microcytotoxicity assay. This suggests that other factors such as humoral immunity may play a significant role.

It is well known that cellular grafts such as neoplastic cells and cells of bone marrow and lymphoid tissue are susceptible to humoral immunity (Winn 1962; Moller and Moller 1962; Stetson 1963) and that skin and organ grafts are rejected by cell-mediated immunity (Brent and Medawar 1967; Roitt 1974). Until recently the role of humoral immunity in the rejection of skin and organ grafts was thought to be of little importance. This view is changing (Carpenter et al 1976). Mullen and Hildemann (1971) and French (1972) have demonstrated antibodymediated damage to rat kidney allografts resulting in decreased graft survival. Abbas et al (1974 a,b) reporting on the histological examination of rat kidney allografts, in the acute phase of rejection, suggested that the acute necrotising arteritis which they found was a manifestation of antibody-mediated rejection. Similar vascular changes have been reported in sheep kidneys perfused with antigraft antibody (Pederson and Morris 1974). Busch et al (1976) have reported three different patterns of rejection of human renal allografts, one of which appears

to be antibody-mediated. Evidence that humoral immunity plays an important role in the rejection of isolated islets has been presented by Naji et al (1975). They showed that islet allografts established in a tolerant host are rejected after injection of donor specific antibody. It is also of interest that the speed of islet rejection correlates with second-set rejection which is predominantly antibody-mediated (Carpenter et al 1976).

Although in our donor-host combination the survival of kidney, heart and skin allografts was very similar (Table IV) this is not always the case. Van Bekkum et al (1969) demonstrated a significant difference in survival of kidney and heart allografts in untreated Wag recipients of Brown Norway grafts. In the pig, orthotopic cardiac allografts are rejected more aggressively than kidney allografts and both survived longer than skin allografts (Calne 1976). Pig liver allografts may survive indefinitely no matter how major the histoincompatibility (Calne et al 1969). The properties of a graft which determines its susceptibility to rejection are undefined. The antigen density on the graft may be important (Calne 1976), but it could also be related to the presence of a vascular endothelium, as vascularised organ grafts tend to survive longer than non-vascularised grafts.

In view of these results it cannot be assumed that isolated islets will behave in a similar way to other grafts in immunosuppressed recipients. If anything, it would appear that the use of specific and non-specific immunosuppression would be less successful for prolonging the survival of isolated islet allografts than for organ allografts. This will be discussed in the next chapter.
CHAPTER IV

METHODS OF ABROGATING THE

REJECTION OF ISLET ALLOGRAFTS

- 1. INTRODUCTION
- 2. HISTOCOMPATIBILITY BARRIER
- 3. ISLET IMMUNOGENICITY
- 4. <u>IMMUNOLOGICALLY PRIVILEGED SITES</u>

5. IMMUNOSUPPRESSION

- a) <u>Non-specific</u>
 - i) Azathioprine and Prednisolone
 - ii) Antilymphocyte serum
 - iii) Cyclophosphamide
 - iv) Carrageenan and Silica
- b) <u>Specific</u>
 - i) Passive Enhancement
 - ii) Active Enhancement
- 6. <u>CONCLUSION</u>

METHODS OF ABROGATING THE REJECTION OF ISLET ALLOGRAFTS

1. INTRODUCTION

The management of diabetes mellitus by transplantation of isolated islets rather than whole pancreas not only has a considerable technical advantage, but also avoids the severe complications associated with transplantation of the exocrine pancreas. It is therefore particularly unfortunate that isolated islets are very susceptible to rejection. In the attempt to minimise the immune reaction between allograft and recipient the immune status of both may be manipulated.

The antigenicity of the graft can be minimised in three ways: by matching the histocompatibility antigens of the donor and recipient, by reducing the immunogenicity of the graft or by transplanting the graft into an immunologically privileged site. Immunoregulation of the recipient can be achieved using non-specific or specific immunosuppression. In this chapter I will discuss the efficacy of these methods in prolonging the survival of isolated islet allografts.

2. HISTOCOMPATIBILITY BARRIER

The important role of histocompatibility is shown by the differing results of isograft (Table I) and allograft (Table III) transplantation. Reckard et al (1973) demonstrated that islets transplanted, intraperitoneally, across a minor histocompatibility barrier had a longer survival than those transplanted across a major histocompatibility barrier. They also showed that a course of antilymphocyte serum (ALS) did not prolong the survival of islets transplanted across the major histocompatibility barrier, whereas it significantly prolonged the survival of those transplanted across the minor histocompatibility barrier. Beyer and Friedman (1978) have reported similar findings. When they transplanted islets, intraportally, from Wistar-Furth to Lewis rats, a major histocompatibility barrier, graft survival was increased by recipient treatment with antithymocyte globulin (ATG) from a mean of two days to a mean of 12 days, whereas when transplanting islets from Fisher to Lewis rats, a minor histocompatibility barrier, similar treatment produced an indefinite graft survival.

The results of histocompatibility matching in the rat (Ag-B) are certainly not uniform. White and Hildemann (1968) have shown that Fischer (Ag-B1) skin grafts were rejected by Lewis (Ag-B1) recipients within 12 days whereas kidney grafts usually survived indefinitely. Using the same model Freeman and Steinmuller (1969) found that heart grafts were rejected within 16 days. Salaman (1971) transplanted renal allografts between AS and AS2 rats which differ at the major histocompatibility locus and found that 70% of AS2 recipients of AS kidneys survived more than 50 days whereas all AS recipients of AS2 kidneys died within 18 days. Skin grafts exchanged between the strains were rejected rapidly. It would thus appear that the value of histocompatibility matching depends on the type of allograft and the donor-recipient strain combination. Present evidence suggests that minimising the histocompatibility barrier has little effect on islet allograft survival in the untreated rat.

Opinion is divided about the value of histocompatibility matching (Human leucocyte A antigens) in cadaver renal transplantation (Sachs 1976; Vincenti et al 1978). Evaluation of its influence on the results of islet transplantation will have to await the advent of human islet transplantation.

3. ISLET IMMUNOGENICITY

Present attempts to prolong allograft survival by modification of the donor-host immune reaction are aimed at host immunosuppression. However, there is also evidence that the immunogenicity of allografts can be reduced (Billingham 1976).

The maintenance of endocrine tissue in organ culture is of particular interest. Jacobs (1974) and Lueker and Sharpton (1974) have shown that mouse ovarian allografts cultured for 8-12 days and three days respectively, showed a significantly prolonged survival over non-cultured allografts. Lafferty et al (1975) reported reduced mouse thyroid allograft immunogenicity after organ culture for 12 days.

Rat islets have also been maintained in organ culture and their functional viability confirmed by subsequent (Weber et al 1975; Hegre et al 1976). isotransplantation Boyles and Seltzer (1975) were the first to report that the in vitro culture of isolated islets reduced their immunogenicity. They compared the survival of islets maintained in culture for less than four days with those cultured for 14-16 days. The islets were transplanted intraperitoneally between different litters of Sprague-Dawley rats, a very minor histocompatibility difference. The islets cultured for four days failed to function, whereas islets cultured for two weeks had an indefinite survival. Eloy et al (1977) provided further evidence that islet immunogenicity could be influenced by in vitro culture. They reported that after only four days in culture rat islets transplanted across a strong histocompatibility barrier had a significantly prolonged survival. Although these studies are of particular interest they present conflicting evidence. It is surprising that Boyles and Seltzer (1975) had to culture the islets for two weeks in order to achieve successful transplantation across a minor histocompatibility whereas Eloy et al (1977) achieved prolonged graft survival after only four days in culture when transplanting across a major histocompatibility barrier. It is also surprising that the control group in Boyles and Seltzer's study did not become normoglycaemic after transplantation, as this would be expected after transplantation across such a minor histocompatibility barrier.

However, Mandel (1978) has recently reported that foetal mouse islets maintained in culture for 30 days before transplantation under the renal capsule were rejected slowly over two to four weeks. Rejection was assessed histologically. Although these studies provide some evidence that islet immunogenicity can be reduced in culture, this approach to minimising the islet-recipient immune reaction clearly requires further investigation.

Gates and Lazarus (1977) transplanted rabbit neonatal pancreas, encased in 'Nucleopore' chambers, intraperitoneally in rats. These grafts were not rejected during the six weeks that they were in the peritoneal cavity. After their removal hyperglycaemia recurred. This is a further interesting attempt to avoid islet rejection, but these chambers need to be inserted into larger animals in order to assess the possible development of a fibrous capsule, as might be expected in man, with the subsequent alteration in permiability.

4. IMMUNOLOGICALLY PRIVILEGED SITES

The cellular nature of islet grafts renders them technically suitable for transplantation into almost any site. However, Kemp et al (1973b) have demonstrated the functional importance of the site of transplantation. They showed that 850-970 islets transplanted subcutaneously had no significant effect on diabetic isologous rat recipients. A similar number of islets transplanted into the peritoneal cavity lowered the blood sugar but did not return it to normal. However, transplantation of 400-600 islets intraportally reverted the recipients to normoglycaemia. Barker et al (1975) have suggested that not only does the intrahepatic site of transplantation have a physiological advantage, but that it is also immunologically privileged. They showed that rat islets transplanted across a minor histocompatibility barrier (DA \rightarrow ACI) had a median survival of 30.5 days if transplanted intraportally and a median survival of only 11.5 days if transplanted intraperitoneally.

Previously Reckard and Barker (1973) and Reckard et al (1974) had reported that isolated islet allografts transplanted under the renal and splenic capsules, intraportally, subcutaneously, intraperitoneally, intramuscularly and into the testis sensitised the recipient so that a subsequent donor strain skin graft was rejected in an accelerated fashion. This evidence contradicts their belief that the liver is an immunologically privileged site. Adler et al (1977) have also suggested that the intraportal site of transplantation is immunologically privileged for islets. Skin grafts applied to rats sensitized with intraportal islets and subcutaneous islets had a mean survival of 7 and 6.2 days respectively. Although they report these figures to be statistically significant, the small difference between them and the fact that skin graft rejection in both experiments was significantly faster than in unsensitized rats provides poor evidence of reduced immunogenicity of intraportal islets. Finch et al (1977) have compared the survival of isolated islet allografts transplanted intraportally and intrasplenically and have found no significant difference in survival.

There is at present no good evidence that the advantage of the intrahepatic site of transplantation is other than physiological, as demonstrated by Kemp et al (1973b). The findings of Barker et al (1975) could be interpreted as demonstrating a physiological rather than an immunological advantage, viz, the functional reserve of the intrahepatic islets enables rejection to progress for longer before it becomes biochemically evident, as shown by a rise in blood Prolonged normoglycaemia might have been achieved sugar. after intraperitoneal transplantation if a much larger number of islets had been transplanted. Finch and Morris (1977a) have demonstrated the importance of functional islet reserve in immunosuppressed rats. Immunosuppressed recipients of 1500 to 3000 intraportal islets were

normoglycaemic for significantly longer than recipients of 600-800 islets.

The testis and omentum in guinea-pigs have also been reported as being immunologically privileged for islets (Ferguson and Scothorne 1972). Histological evidence of survival was reported up to 11 weeks after transplantation. Although the testis in certain circumstances is an immunologically privileged site (Barker and Billingham 1973), the report of Reckard and Barker (1973) that islet allografts injected into the testis sensitized the recipient, would suggest that it is not privileged for islets. Further evidence of both histological and functional survival are required in support of Ferguson and Scothorne's results.

It can be concluded that at present there is no good evidence of an immunologically privileged site for isolated islet transplantation.

5. IMMUNOSUPPRESSION

The present success of human kidney transplantation has been achieved through the use of potent non-specific immunosuppressive agents such as azathioprine, prednisolone and cyclophosphamide. Specific immunosuppression has been attempted (Batchelor et al 1970; French and Batchelor 1972), but without success. In this section I will discuss the efficacy of both non-specific and specific immunosuppression on the survival of isolated islet allografts in rats. Although the results of animal experiments cannot be extrapolated to man, the behaviour of islets in immunosuppressed rats can be compared with the behaviour of other grafts in similarly treated recipients. These findings should give an indication of the prospects of prolonging islet allograft survival in man.

a) Non-specific Immunosuppression

i) Azathioprine and Prednisolone

These two agents are the most commonly used in human renal transplantation. Azathioprine is an antimetabolite which is thought to act by blocking the differentiation of lymphocytes and thus interfering with host sensitization (Lance 1976). The action of prednisolone is complex and poorly understood, but it is very effective at prolonging the survival of allografts (Lance 1976).

Four diabetic AS rats were treated with 4 mg/kg of azathioprine after receiving an (AS x Aug)F₁ islet allograft (Table V). The first dose was given intraperitoneally immediately after transplantation and this was followed by daily intraperitoneal doses for five days. All the grafts were rapidly rejected.

A group of eight diabetic AS rats were then similarly treated with azathioprine 4 mg/kg and prednisolone 4 mg/kg after islet transplantation (Table V). These grafts were rejected with equal rapidity. From these results it was clear that neither azathioprine nor a combination of azathioprine and prednisolone in a dose of 4 mg/kg prolonged islet allograft survival in our donor-recipient combination. On the contrary, the results suggested that these agents were in some way toxic to isolated islets.

Husberg (1973) has shown that 30 mg/kg of azathioprine had no effect on the cell-mediated cytotoxicity of thoracic duct lymphocytes from Brown Norway (BN) rat recipients of Wistar Furth (WF) kidneys. He also found that there was only a moderate reduction in cytotoxic antibodies in the serum of the treated recipients. Further evidence of the inability of azathioprine to achieve immunosuppression in rats was reported by Shehadeh et al (1970) and Winearls et al (1978). Shehadeh et al (1970) treated Lewis recipients of (L x BN)F₁ kidneys with 8 mg/kg/day to 40 mg/kg/day of azathioprine and found no significant renal function at seven days. Winearls et al (1978) found no significant prolongation of survival of Lewis recipients of (DA x Lewis) F_1 kidneys treated with 4 mg/kg/day of azathioprine. In contrast, Tinbergen (1968) reported a significantly prolonged mean survival of Wag recipients of BN kidneys treated with 4 mg/kg/day of azathioprine. The range of survival of his control group was 9 to 55 days, which suggests the histocompatibility barrier was minor and this might explain his good results. Van Bekkum et al (1969) using the same

<u>Treatment</u>	<u>No. of Rats</u>	<u>No. of Days Before</u> <u>Rejection</u>
Control	10	0, 0, 0, 2, 2, 3, 3, 3, 5, 5
Azathioprine (4 mg/kg)	4	0 , 0 , 0 , 2*
Azathioprine (4mg/kg) Prednisolone(4mg/kg)	8	0, 0, 0, 0, 0, 0, 0, 0.

* Died Normoglycaemic

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<u>Table V</u> $(AS \times Aug)F_{1} \longrightarrow AS$. Islet allograft survival in recipients treated with azathioprine or a combination of azathioprine and prednisolone. rat model as Tinbergen (1968), found that cardiac allografts in recipients treated with 4 mg/kg/day of azathioprine survived longer than the controls, but not as long as renal allografts in treated recipients. Marquet and Heystek (1975) also using the same rat model, found that isolated islet allografts in similarly treated recipients survived longer than heart allografts but shorter than kidney allografts. This is the only rat model in which azathioprine has been reported as an effective immunosuppressive agent in prolonging islet allograft survival.

Rats are steroid sensitive animals (Claman 1972) and treatment of kidney allograft recipients resulted in depressed cell-mediated immunity as shown by a decrease in the number of cells in the thoracic duct lymph and by a decreased cytotoxic capacity of the remaining cells (Husberg (1973). It was therefore hoped that the addition of prednisolone would improve islet allograft survival. Nelken et al (1977) reported no improved survival of islet allografts in recipients treated with prednisolone alone. Finch and Morris (1976) reported similar findings in recipients treated with a dose of azathioprine and prednisolone which prolonged the survival of renal allografts in the same rat model. Salaman et al (1977) demonstrated that azathioprine had no effect on prolonging cardiac allografts, but combined with prednisolone two out of seven allografts had a significantly prolonged survival.

These studies indicate that neither azathioprine nor a combination of azathioprine and prednisolone are particularly effective in prolonging islet allograft survival. Only the

studies of Marquet and Heystek (1975) have shown benefit from treatment with azathioprine, but this could be related to the fact that islets in their untreated recipients survived 7-12 days, much longer than in our model (Table III). The marginal improvement in survival of heart and kidney allografts in treated rats emphasises the difference between the action of these drugs in rats and man. However, the failure to achieve even minimal improvement in islet allograft survival is evidence of their susceptibility to rejection.

In dogs, a more reliable experimental animal for predicting the efficacy of immunosuppressive agents in man, azathioprine and prednisolone significantly prolong the survival of renal allografts (Calne 1967). However, Kolb et al (1978) have recently reported that these agents are ineffective at suppressing the rejection of islet allografts in dogs.

ii) Antilymphocyte Serum (ALS)

Azathioprine and prednisolone do not discriminate between types of lymphocyte. The action of ALS on the other hand is directed against thymus-dependent lymphocytes (Turk and Willoughby 1967) and thus cell-mediated immunity. The action is not absolutely specific in view of the co-operation between T and B lymphocytes (Owen 1976).

Anti-rat-lymphocyte serum was raised in three New Zealand white rabbits. 1×10^8 thymus and lymph node cells taken from AS rats were injected intravenously on days 0, 14 and 26. The rabbits were bled on days 19 and 34. The serum was pooled, inactivated by heating at 56° C for 30 minutes, divided into 1 ml aliquots and stored at -20° C.

Six diabetic AS recipients of $(ASx Aug)F_1$ islets were treated with 1 ml of ALS on days -1, 1, 3 and 5. All the treated recipients rejected the islets as rapidly as the untreated controls (Table VI).

The efficacy of ALS in prolonging islet allograft survival varies considerably. Gray and Watkins (1974) reported indefinite survival of Wistar-Lewis islets in Fischer rats maintained on treatment with ALS. Reckard et al (1973) reported no increased survival of Fischer islets in ALS treated ACI rats. However, transplanting across a minor histocompatibility barrier (ACI -> DA) a five day course of ALS prolonged islet allograft survival from a median of 8 days to a median of 30 days, but in the same donor-host combination similar recipient treatment facilitated indefinite skin graft survival. Vialettes et al (1978), transplanting neonatal islet tissue from Fischer to Lewis rats, reported that antithymocyte serum (ATS) did not prolong graft survival. Finch and Morris (1977b) treated Lewis recipients of DA islets with ALS the day before transplantation and one three and five days after transplantation. Graft survival was prolonged from 2-5 days to 22-32 days.

Beyer and Friedman (1978) demonstrated that the important factor determining the efficacy of ATS in prolonging islet allograft survival was the histocompatibility difference between donor and recipient. The survival of islets transplanted across a major histocompatibility barrier (WF \rightarrow Lewis) was only increased from 2 to 12 days by recipient treatment, whereas indefinite survival of Fischer islets in ATS treated Lewis recipients was achieved.

In view of the susceptibility of islets to humoral

Treatment	No. of Rats	No. of Days Before Rejection
Control	10	0, 0, 0, 2,
		2, 3, 3, 3,
		5, 5
Antilymphocyte	6	0, 0, 0,
Serum		0, 0, 3

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<u>Table VI</u> $(AS \times Aug)F_1 \longrightarrow AS$. Islet allograft survival in recipients treated with antilymphocyte serum.

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immunity it would not be surprising if ALS failed to prolong graft survival. However, it clearly is effective although only across certain histocompatibility barriers. This effect is probably obtained by suppression of both cell-mediated and humoral immunity, the influences on humoral immunity being achieved through its dependence on T and B cell co-operation. This theory is substantiated by the work of Rolstad et al (1974), who have reported that the antibody response in the rat is markedly thymus dependent.

iii) Cyclophosphamide

Whereas the action of ALS is directed towards T lymphocytes, cyclophosphamide has a preferential effect on B lymphocytes (Turk 1973). However, Husberg (1972) has shown that in rats it will depress both cell-mediated and antibody-mediated immunity directed against an allogeneic kidney.

Six diabetic AS rats transplanted with (AS x Aug) F_1 islets were treated with 10 mg/kg of cyclophosphamide. The initial dose given immediately after transplantation was injected intravenously. Thereafter daily intraperitoneal doses were given for five days. All the grafts were rapidly rejected (Table VII).

Finch and Morris (1977a) reported a mean increase in survival of (DA x Lewis) F_1 islets in Lewis recipients, treated with 10 mg/kg/day of cyclophosphamide, of 4.2 to 6.8 days. Vialettes et al (1978) treated Lewis recipients of Fischer rats with 25 mg/kg of cyclophosphamide after transplantation. Five rats rejected their grafts before

Treatment	No. of Rats	No. of Days Before Rejection
Control	10	0, 0, 0, 2, 2, 3, 3, 3, 5, 5
Cyclophosphamide (10mg/kg)	6	0, 0, 0, 0, 0, 0.

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Table VII	(AS x Aug)F.	\longrightarrow AS.	Islet allograft
	survival in	'recipients	treated with
	cyclophospha	amide.	

day one and the other five were rejected between 6 and 11 days.

It is clear from these results that the preferential effect of cyclophosphamide on B lymphocytes, and thus humoral immunity, did not have much beneficial effect on graft survival. In our rat model cyclophosphamide was as ineffective as ALS, but in the model used by Finch and Morris (1977 a,b) ALS was significantly more effective than cyclophosphamide. Their results and those of others have clearly shown that ALS is the most effective non-specific immunosuppressive agent for prolonging islet allograft survival.

iv) Carrageenan and Silica

In the previous sections I have examined the efficacy of those immunosuppressive agents which do not discriminate for lymphocytes, those which do, and those which discriminate between lymphocyte subpopulations. As the immune response is a complex interaction between T lymphocytes, B lymphocytes and macrophages (Roitt 1974, Owen 1976), I will discuss in this section the influence of macrophage suppression on islet allograft survival (Nash and Bell 1978).

Carrageenan, a polysaccharide extracted from the marine alga Chondrus Crispus, and silica, a major constituent of the Earth's crust, which are both known to lyse macrophages (Allison et al 1966), were used to suppress macrophage function. Purified lambda carrageenan (Marine Colloids, Springfield, N.J.) was given in a single dose of 5 mg/kg intravenously either five days pre-transplantation, two days pre-transplantation, or immediately after transplantation.

Silica dust (Dorentup Quartz No. 12. 1-5u) was given in a single dose of 50 mg/100 g intraperitoneally either six days pre-transplantation or two days pre-transplantation. The silica was suspended in 1 ml of normal saline and exposed to 30 minutes ultrasonic vibration before injection.

Only one recipient of the 13 treated with carrageenan had prolonged graft survival, the graft functioning for longer than 100 days. The remainder showed no increased graft survival (Table VIII).

Silica pretreatment was much more effective in prolonging islet allograft survival in that nearly half of the grafts functioned for longer than 100 days; treatment six days pre-transplantation being much more effective than treatment two days before transplantation (Table IX).

The role of macrophages in the immune response, although important, is not nearly so clearly defined as the role of T and B lymphocytes which express cell-mediated and humoral immunity respectively. It is probably for this reason that the lymphocyte has been the main target for immunosuppression and the macrophage has been neglected.

Lambda carrageenan has been shown to be toxic to macrophages in vitro (Allison et al 1966; Catanzaro et al 1971), but has a number of other actions including the inhibition of the complement system and the activation of Hageman factor (Di Rosa 1972). Reports about its action in vivo are contradictory. Everson et al (1978) have shown that a single dose of lambda carrageenan given immediately after transplantation will significantly prolong the survival of cardiac allografts in the rat, but they were unable to demonstrate in vivo suppression of macrophage function using the carbon clearance test.

Treatment	ъ.	No. of Rats	No. of Days Before
			Rejection
Control		10	0, 0, 0, 2.
			2, 3, 3, 3,
			5, 5
Carrageenan (5mg/kg)	Day O	5	0, 2, 2, 3, >100
(2mg/ng/	Day - 2	3	0, 2, 5
	Day - 5	5	0, 0, 0
			2, 2
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<u>Table VIII</u> (AS x Aug)F \longrightarrow AS. Islet allograft survival in recipients treated with carrageenan.

Treatment		No. of Rats	No. of Days Before Rejection
Control		10	0, 0, 0, 2, 2, 3, 3, 3, 5, 5
Silica 50 mg/100g	Day - 2	10	0, 2, [*] 3, 3, 4, 5, 13 [*] , >100 >100, >100
	Day - 6	6	3, 5, >100 >100, >100, >100

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* Died Normoglycaemic

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Rios and Simmons (1972) have shown that pretreatment with carrageenan will prolong the survival of skin allografts in the mouse and that parallel treatment with a macrophage stabilising agent poly-2-vinylpyridine N-oxide (PVNO) abrogates the effect. Aschheim and Raffel (1972) on the other hand have shown that pretreatment with intraperitoneal lambda carrageenan actually increased the uptake of ⁵¹Cr labelled sheep red blood cells by mouse peritoneal macrophages. Our results show that although it is possible for lambda carrageenan to prolong islet allograft survival, it is not very effective, and in view of the results achieved with silica it would appear that carrageenan in the dose used is not a very potent antimacrophage agent.

Silica is a specific macrophage toxin (Kessel et al 1963) which causes lysosomal destruction of macrophages in vitro (Allison et al 1966) and inhibits their activity in vivo (Levy and Wheelock 1975). Pearsall and Weiser (1968) and Rios and Simmons (1972) demonstrated a prolongation of skin graft survival in mice of four to five days if the recipients were pretreated with silica. Our results demonstrate the efficacy of macrophage suppression on islet allograft survival. It is of particular interest that a single dose of silica resulted in indefinite survival of those grafts not rejected by five days. It seems unlikely that this is due to persistent macrophage suppression, but more likely that macrophage suppression has enabled the grafts to survive long enough for a state of enhancement to develop.

Macrophages make up a substantial part of the cellular infiltrate of acutely rejecting organ grafts, but they take a number of days to infiltrate into the graft and their role is undefined (Tilney et al 1976). They have also been reported to play a prominent role in the rejection of isolated islet allografts (Kolb et al 1978). The accessibility of isolated islets may explain the rapidity of their destruction. It is of interest to compare the difference between organ allografts and isolated islet allografts with primary tumours and metastases, as metastases appear more susceptible to immune destruction than primary tumour (Milas et at 1974), and the suppression of macrophages greatly increases the number of metastases and vice versa (Sadler et al 1977).

Naji et al (1975) have demonstrated the vulnerability of isolated islets to immune serum and evidence that macrophages are responsible for antibody-mediated graft rejection has recently been presented by Denham et al (1978). They detected killing of labelled Hooded rat lymphoma cells, transplanted intraperitoneally into August rats primed with August anti-Hooded rat serum, within three hours of transplantation. Recipient pretreatment with Iota carrageenan abolished this effect. Unfortunately, as already discussed, carrageenan has a number of actions and its toxicity to macrophages appears weak. This study needs to be repeated using silica instead of carrageenan as its action is specific for macrophages. Further evidence that macrophages could be the effector cells responsible for isolated islet destruction is demonstrated by their ability to kill foreign cells in vitro (Granger and Weiser 1964; Evans and Alexander 1972) and in vivo (Chambers and Weiser 1972; Roos and Dingemans 1977).

In conclusion it appears that macrophages are important in the rejection of isolated islet allografts and that macrophage suppression may prove an effective means of prolonging islet allograft survival.

b) Specific Immunosuppression

At present the success of human renal transplantation is dependent on non-specific immunosuppressive agents, but unfortunately these agents cause a wide range of complications (Lance 1976). Hopefully, specific immunosuppression defined as immunosuppression directed only at the lymphocyte clones responsible for the rejection of a particular graft, i.e. antigen specific immunosuppression (Fabre 1976), will achieve an improvement in potency and safety. Such immunosuppression may be achieved by treating the graft recipient with antigraft antiserum - passive enhancement, by pretreating the graft recipient with cells or cell extracts carrying the same antigenic incompatibilities as those on the allograft active enhancement, or by a combination of both methods (Batchelor and Welsh 1976).

i) Passive Enhancement

Donor specific alloantibody, or enhancing serum, was raised in AS rats by six weekly intraperitoneal injections of 1-2x10⁶ August thymus and lymph node cells. Additional booster injections were given monthly thereafter. The cytotoxicity of the pooled antiserum was assayed using August lymphocytes and guinea pig complement in the trypanblue dye exclusion test (James 1973). The antiserum gave a consistent cytotoxicity titre of 1:256 or above, with an end point of 50% cells killed. Recipients to be enhanced received 1 ml of serum intravenously immediately after transplantation, 1 ml intraperitoneally the following day, and 0.5 ml intraperitoneally for a further three days (French and Batchelor 1969).

Ten diabetic AS rats transplanted with $(ASx Aug)F_1$ islets received a course of enhancing serum (Table X). All but three grafts survived longer than the controls, and four grafts survived longer than 100 days. These four recipients remained normoglycaemic indefinitely (Figure 18).

Immunological enhancement is clearly an effective method of prolonging islet allograft survival, but the precise mechanism of its action is unclear. Enhancing antibody could have a peripheral or a central influence on the immune response. The peripheral effect could be to prevent antigen recognition or to block the action of the effector cells. Morris and Lucas (1971) demonstrated diminished uptake of labelled antigraft-antibody by enhanced rat kidneys 2, 15 and 265 days after transplantation and suggested that this was due to the coating of antigen sites on the enhanced graft by endogenously produced antibody. However, Fine et al (1973) and French (1973) using a different strain combination, showed that enhanced kidney allografts had an undiminished number of free antigen sites. Further evidence against an afferent blocking mechanism was presented by Fabre and Morris (1973). They demonstrated that very small volumes of enhancing serum were effective in prolonging renal allograft survival.

An efferent blocking mechanism in the maintenance of a functioning enhanced graft was suggested by Bowen et al (1974). They demonstrated the failure of adoptive immunization

Treatment	No. of Rats	No. of Days Before Rejection
Control	10	0, 0, 0, 2 2, 3, 3, 3, 5, 5
As anti August enhancing serum	10	2, 2, 2, 6, 7⁺, 11, >100, >100 >100, >100

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* Died Normoglycaemic

<u>Table X</u> $(AS \times Aug)F_{1} \longrightarrow AS.$ Islet allograft survival in recipients treated with enhancing serum.

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Fig. 18 (AS x Aug)F \longrightarrow AS. Normoglycaemia achieved in a recipient treated with enhancing serum. This rat died normoglycaemic 227 days after transplantation.

and parabiosis with hyperimmune syngeneic partners to abrogate long-term enhancement of rat kidney allografts. Blocking factors in the serum of rats bearing an enhanced graft have been reported by a number of workers (Stuart et al 1971; Strom et al 1975). The nature of these blocking factors is uncertain, but they could be antigen-antibody complexes (Myburgh and Smit 1975; Marquet et al 1976) or anti-idiotypic (antireceptor) antibodies (McKearn et al 1974; Binz and Wigzell 1976). Hutchison and Zola (1977) on the other hand have suggested that antigen reactive cells bind with donor antigen and enhancing antibody and are opsonised and removed by the host macrophages. In this way the effector cells are prevented from reaching the graft. Later, endogenously synthesized antibody replaces the enhancing antibody to maintain the opsonization of antigen reactive cells. If blocking factors are important in the maintenance of enhancement, serum from rats with enhanced grafts should be highly effective as enhancing serum. Fabre and Morris (1972) reported that serum transfer experiments were ineffective at prolonging kidney allograft survival in their donor-host combination. Tilney and Bell (1974) on the other hand found that serum from rats bearing an enhanced kidney or heart was an effective enhancing serum.

It is difficult to explain why, under apparently standard conditions, only four out of ten islet allografts had a prolonged survival in recipients treated with enhancing serum, but in view of the possibility that conditions in the four recipients with functioning grafts

facilitated the production of blocking factors, serum transfer experiments were performed using pooled serum from these four rats (Nash et al 1978).

Five diabetic AS rats were treated with this serum after receiving an $(AS \times Aug)F_1$ islet allograft. The dose and route of administration were the same as used for conventional enhancing serum. Only one graft had a prolonged survival, surviving longer than 100 days (Table XI). Although this showed that the serum was capable of prolonging islet allograft survival, the results were no better than those achieved using conventional enhancing serum. This is strong evidence against the presence of blocking factors.

The mechanism of enhancement is undoubtedly complex, but as a peripheral action of enhancing serum has not been clearly established, it is likely that it has a central action (French and Batchelor 1972; Brent and French 1973). In a recent review Batchelor and Welsh (1976) suggested that the mechanism involved a modification of the normal interactions between specific lymphocyte subsets.

Whatever the changes that take place in the recipient, the nature of the graft is also important in the establishment of enhancement. In a recent study (Nash et al 1976) we compared the survival of isolated islet, heart, kidney and skin allografts transplanted into recipients treated with a course of enhancing serum (Table XII). Whereas the majority of heart and kidney allografts had a prolonged survival, only 40% of the islet allografts survived longer than 100 days. Skin graft survival was only increased by four or five days. In an attempt to explain the difference

Treatment	No. of Rats	No. of Days Before Rejection
Control	10	0, 0, 0, 2, 2, 3, 3, 3, 5, 5
Serum from AS rats bearing an enhanced (AS x AUG) F ₁ islet graft	5	0, 2, 2, 2, >100

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<u>Table XI</u> (AS x Aug)F \longrightarrow AS. Islet allograft survival in recipients treated with serum obtained from rats bearing a functioning, enhanced, islet allograft. .

Tissue	No. of Rats	No. of Days Before Rejection
Islets	10	2, 2, 2, 6, 7*, 11, >100, >100 >100, >100
Kidney	7	> 100 (7)
Heart	11	35 ⁺ , 55, 58, 61, > 100 (7)
Skin	8	9, 10, 11, 12, 12, 13, 13, 14

* Died Normoglycaemic

+ Died with functioning Heart Allograft

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<u>Table XII</u> (AS x Aug)F \longrightarrow AS. Comparative survival of kidney, heart and skin allografts, in recipients treated with enhancing serum.

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in effectiveness of enhancement between skin and kidney allografts, Fabre and Morris (1975) have suggested that either renal allografts are more resistant to rejection than skin grafts, or that skin grafts are susceptible to a wider pool of reactive lymphocytes. This hypothesis could not explain the results of islet enhancement, as just under a half had a prolonged survival similar to the organ grafts, whereas the remainder behaved in a similar way to the skin grafts. Finch and Morris (1976) using a different rat model have also reported the efficacy of passive enhancement in the suppression of islet rejection and their results, like ours, were not as good as would be expected for organ grafts.

Poole et al (1976) attempted to prolong the survival of (AS x Aug) F_1 leg allografts on AS rats using passive enhancement, but graft survival was no better than in the untreated recipients. They then showed that leg allografts transplanted onto AS rats bearing a well established functioning enhanced (AS x Aug) F_1 kidney had a significantly prolonged survival. This experiment highlighted the difference between the induction and maintenance phase of enhancement (Batchelor and Welsh 1976). Mullen et al (1973) demonstrated a similar phenomenon using skin grafts. In a further attempt to improve the results of islet allograft enhancement we transplanted islets into diabetic recipients bearing functioning enhanced cardiac allografts (Table XIII) (Nash et al 1978). All the grafts were rejected by the second day no matter how long the interval between cardiac

Experiment No.	No. of Days after Heart Transplant	No. of days Before Rejection of Islet Allograft
72	140	2
73	126	0
113	120	0
143	78	2*
114	64	0
144	60	0
147	52	0
122	21	2

* Died Normoglycaemic

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Table XIII	$(AS \times Aug)F_1 \longrightarrow AS.$ Islet allograft
	survival in recipients bearing a functioning, enhanced (AS x Aug) F_1 heart allograft.

and islet transplantation. The mechanism of their rejection is unclear but the rapidity would suggest that it was antibody-mediated.

Finch and Morris (1977b), also in an attempt to improve the efficacy of enhancing serum in prolonging islet allograft survival, treated recipients with a combination of enhancing serum and ALS. Batchelor et al (1972) and Fabre and Morris (1974) had previously shown that enhancing serum and ALS acted synergistically in suppressing rejection of rat renal allografts. Unfortunately Finch and Morris (1977b) were unable to demonstrate any such effect on the rejection of isolated islet allografts.

In view of the difficulty of prolonging islet allograft survival using non-specific immunosuppressive agents, the results achieved using passive enhancement are encouraging. However, these results have only been achieved in rats by transplantation between the first generation hybrid and parent strains and are not as good as those obtained when transplanting organ grafts. It would thus appear that if an effective method of achieving passive enhancement in clinical renal transplantation is developed, it cannot be taken for granted that it will be equally effective in prolonging islet allograft survival.

ii) Active Enhancement

Active enhancement of recipients of renal allografts in the rat have resulted in prolonged graft survival (Stuart et al 1968). However, the efficacy of recipient

pretreatment with donor strain antigens, together with a short course of ALS, in prolonging skin graft survival in incompatible mice demonstrated the potential of active

enhancement (Brent et al 1973). The ability of this technique to prolong skin graft survival suggested that it would also be effective in preventing the rejection of isolated islets.

Panijayanond and Monaco (1974) pretreated (C57BL x Ajax)F1 mice with ALS and donor bone barrow before transplanting C3H/He islets. The mean graft survival of three mice was nine weeks. A similar treatment regime had previously been shown to prolong skin grafts in the same donor-recipient combination (Wood et al 1972). Nelken et al (1977) reported a prolonged survival of Wistar-Furth islets in Lewis recipients treated with liver extract, Bordetella pertussis and ALS, a treatment regime that Pinto et al (1974) have shown to be highly effective in prolonging the survival of skin grafts in incompatible mice. The mean survival of the islets in the treated rats was 37.7 days and only 2 days in the untreated group. Recently Vialettes et al (1978) have shown that the survival of Fischer islets in Lewis recipients was prolonged when treated with a combination of soluble membrane antigen from Fischer spleen cells and ATG. Although the increase in survival was only marginal, this treatment regime was significantly better than ATG, cyclophosphamide or soluble antigen given alone.

These results show that active enhancement is effective in prolonging islet allograft survival. The best indication of its success is shown in the study reported by Nelken et al (1977). ALS, which appears to be the best non-specific immunosuppressive agent in suppressing the rejection of
isolated islets, only prolonged islet survival from two to 13 days, whereas active enhancement prolonged islet survival to 37.7 days.

6. CONCLUSION

These studies show that in our rat model standard non-specific immunosuppressive agents were ineffective at preventing islet allograft rejection. Other groups have found ALS effective but only in certain donor-host combinations. Specific immunosuppression on the other hand has been much more effective, although less so than for organ grafts. As 50% of cadaver renal allografts are lost, mainly due to acute rejection, in the year after transplantation the prospects for islet transplantation are not encouraging unless other immunosuppressive techniques can be developed. Successful human islet allotransplantation has not been reported although it has been attempted (Najarian et al 1977). This failure could be due to the susceptibility of islets to rejection.

The role of macrophages in the rejection of islet allografts is of particular interest as their suppression had a marked effect on graft survival. Present immunosuppression is directed at T and B lymphocytes. However, its efficacy could probably be greatly enhanced by including an anti-macrophage agent, as all three cell types play an important role in the immune response.

CHAPTER V

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HUMAN ISLET TRANSPLANTATION

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HUMAN ISLET TRANSPLANTATION

Whole pancreas and isolated islet transplantation have been undertaken in man. The results of whole organ transplantation are, however, disappointing. Only two patients have survived longer than a year, the longest surviving just over four years (Connolly 1978). The greatest problem encountered in whole organ transplantation has been the drainage of pancreatic juice from the exocrine Initially, the proximal end of the donor's pancreas. duodenum was oversewn and the distal end exteriorised as a cutaneous duodenostomy (Lillehei et al 1970). Later. the same group developed a technique of draining the distal duodenum into a Roux-en-Y jejunal loop in the recipient (Lillehei et al 1970). Gliedman et al (1973) developed a further improvement in pancreatic drainage by anastomosing the pancreatic duct, end to end, to the distal ureter. Unfortunately none of these methods has been very successful. It was for this reason that isolated islet transplantation appeared to have a great advantage over whole organ transplantation.

The first human islet transplants were undertaken by Najarian et al (1977). They transplanted seven diabetic patients who were receiving standard immunosuppressive treatment for previously transplanted renal grafts. The patients received either collagenase digested and Ficoll gradient isolated adult islets, or collagenase digested infant pancreas. The islet tissue was transplanted intraportally or intraperitoneally. Although the insulin requirements in the post-operative period were reduced, it was not possible to withdraw treatment from any of the patients. The role of rejection in graft failure was difficult to evaluate as the inability to achieve normoglycaemia could have been due to insufficient islets being transplanted. A point of particular interest was that no complication arose as a result of intraportal injection of islet tissue.

A number of workers have isolated viable islets from human pancreas (Sutherland et al 1974; Lawson and Poutala 1974; Anderson et al 1976; Najarian et al 1977), but the major difficulty encountered has been in obtaining a sufficient yield for transplantation. In the rat we required six donors to obtain more than 500 islets, but, even under standard conditions, such a yield was not always obtained. In an attempt to improve islet yield Mirkovitch and Campiche (1976) developed a technique of islet preparation and transplantation in the dog which avoided islet separation on a Ficoll gradient. After the pancreas had been chopped into small pieces and digested with collagenase it was injected directly into the spleen. Their results were promising and led others to extend these studies. Kretschmer et al (1978) reported that after mechanical chopping (Matas et al 1976b), approximately 50% of the insulin and amylase content was lost and that further loss occurred during collagenase digestion. After 15 minutes digestion 18% of the original insulin content was recovered and after 20 minutes digestion only 8% was recovered. However after transplantation the pancreas

digested for 20 minutes produced better diabetic control. These studies highlighted the important balance between tissue dispersal and the amount of viable islet tissue recovered for transplantation. In the same study they compared the results of intrasplenic and intraportal transplantation and found that in dogs the former produced better control of diabetes. A further unsatisfactory finding after intraportal transplantation was the development of portal hypertension.

Najarian's group have recently extended their studies to man (Sutherland et al 1978; Najarian et al 1978). Three patients with severe debilitating chronic pancreatitis were treated by total pancreatectomy. Immediately after excision the pancreas was prepared for autotransplantation by mechanical chopping and collagenase digestion. The dispersed tissue was then injected into the portal vein. A transient rise in portal pressure was noted during the infusion and post-operatively minor changes in the liver function tests occurred, but these had returned to normal within one month of transplantation. The patients did not develop diabetes. These studies are of particular importance as they have demonstrated the feasibility of transplanting dispersed pancreatic tissue into the portal vein and have shown that using this technique of islet preparation, sufficient tissue is obtained from a single pancreas to control diabetes.

Clearly major advances have been made in the preparation of pancreatic tissue for islet transplantation. As further improvements are made it may even become possible to use a

single donor pancreas for more than one recipient. Maximal utilisation of islet tissue will require the development of reliable methods of islet preservation. Andersson et al (1976) have maintained isolated human islets in tissue culture for one to three weeks. Frankel et al (1976) have stored mouse islets for up to five weeks at 8° C and Kemp et al (1977) have reported in vivo viability of rat foetal islets after preservation at -196°C. These studies are encouraging and it would appear that a reliable technique of islet preservation can be developed.

The susceptibility of isolated islet allografts to rejection remains the major obstacle to their use in the management of diabetes. The results of the studies presented in this thesis suggest that the available immunosuppressive regimes are inadequate in suppressing the rejection of isolated islets. A significant recent advance in immunosuppression has been the discovery of the potent immunosuppressive activity of the fungal metabolite Cyclosporin A. This agent is highly effective in suppressing the rejection of heart allografts in rats (Kostakis et al 1977), renal allografts in dogs (Calne and White 1977), renal allografts in rabbits (Green and Allison 1978) and heart allografts in pigs (Calne et al 1978a). Preliminary results of a study in human renal transplantation suggest that it is also effective in man (Calne et al 1978b). The mode of action of Cyclosporin A is unclear, but it appears to have a specific effect on antigen reactive cells (White et al 1978). The success of specific immunosuppression in suppressing the rejection of isolated islets in animal

models suggests that the donor specific action of Cyclosporin A may provide an effective method of prolonging islet allograft survival.

A great improvement in the management of diabetes mellitus has been made since the discovery of insulin in 1921. However the development of long-term complications in diabetic patients highlights the inadequacy of present treatment. Evidence suggests that these complications can be prevented by transplantation of isolated islets. Although transplantation is not available, the rapidity of advances in this field suggests that this will be achieved in the near future.

APPENDIX A

THE COLORIMETRIC DETERMINATION

OF GLUCOSE IN WHOLE BLOOD

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THE COLORIMETRIC DETERMINATION

OF GLUCOSE IN WHOLE BLOOD

Principle

	Acid		
Ortho-toluidine + Glucose	·>	Coloured	Complex
(Colourless)	Heat	(Blue-6	green)

In the presence of heat and acid o-Toluidine reacts readily with glucose to form a coloured complex. The intensity of the colour is measured with a photoelectric spectrophotometer (Unicam SP1800 Ultraviolet Spectrophotometer linked to a Unicam AR25 Linear Recorder) at wavelength 635 nm, and is proportional to the glucose concentration.

Reagents

- 1. o-Toluidine reagent: This contains 6% o-Toluidine $(w/_v)$ in glacial acetic acid. Thiourea is added as a stabiliser.
- 2. Trichloracetic acid solution (TCA): 3% (w/v)
- 3. Glucose standard solutions:
 A stock solution standardised at 50 mmol/litre containing benzoic acid as a preservative.
 From this solution the following standard

solutions are made to prepare a

calibration curve:

2.5 mmo1/L	5.0 mmo1/L
7.5 mmol/L	10.0 mmol/L
12.5 mmol/L	17.5 mmol/L
22.5 mmo1/L	25.0 mmo1/L

Specimen Collection and Preparation

- Between 0.3 and 0.5 ml of blood is taken from the tail of the anaesthetised rat, directly into a tube containing sodium fluoride and oxalate. Oxalate as an anticoagulant and fluoride to avoid glycolysis.
- 2. To deproteinise the blood, pipette 0.2 mls of each specimen into a centrifuge tube. Add 1.8 mls of 3% TCA. Mix well and allow to stand for approximately 5 minutes. Then centrifuge for 10 minutes at 2000 rpm until the supernatant is clear. The supernatant is removed and used for immediate glucose estimation or stored frozen in a glass specimen bottle for glucose evaluation at a later date.

Method

- Prepare a blank by adding 0.1 ml of distilled water to 0.9 ml of 3% TCA.
- 2. Prepare the 8 standards by adding 0.1 ml of each standard to 0.9 ml of 3% TCA.
- Prepare the test samples by taking 1 ml of the supernatant obtained above.

- 4. To each tube add 5.0 ml o-Toluidine
 reagent. Mix well and place in boiling
 water for 12 minutes.
- 5. Quickly remove all the tubes and cool to room temperature by placing in tap water for three minutes.
- 6. Transfer contents of tubes to Cuvettes and read absorbance at 635 nm using the blank as reference. Readings must be completed within 30 minutes.

Calculation of Results

- Plot the absorbance of each standard against the glucose concentration on linear graph paper. This is a straight line passing through the origin.
- 2. Read off the results of the test samples from this graph.

APPENDIX B

THE RADIOIMMUNOASSAY OF INSULIN

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APPENDIX B

THE RADIOIMMUNOASSAY OF INSULIN

(Insulin RIA kit, The Radiochemical Centre, Amersham, Bucks.)

Principle

Insulin is assayed by a double antibody radioimmunoassay using rat insulin standards.

Guinea-pig anti- insulin antibody	+	Rabbit ant pig gamma antibody	i-Guinea globulin _	→	Insulin binding reagent	
Insulin binding reagent	+	Unknown insulin ⁺	Iodinated insulin		<pre>Insoluble complex</pre>	

The assay depends upon the competition between unlabelled insulin and labelled (^{125}I) insulin for the binding sites on the insulin binding reagent. The proportion of labelled insulin bound to the insulin binding reagent will vary inversely with the concentration of unlabelled insulin in the sample under investigation. The quantity of labelled insulin in the insoluble complex is measured by counting the precipitate on a gamma counter. A standard curve is obtained using a number of rat insulin standards.

Reagents

1. Iodinated (^{125}I) insulin solution in stabilized phosphate buffer containing not more than 10 uCi 125_T .

- 2. Freeze-dried insulin binding reagent.
- 3. Freeze-dried rat insulin standard (Novo, Copenhagen, Denmark). Reconstitute the freeze-dried insulin with buffer to 200 ng/ml stock solution from which six standards were made;

0.4	ng/ml	1	ng/m1
2	ng/ml	3	ng/m1
4	ng/ml	6	ng/ml

- 4. Freeze-dried buffer.
- 5. Freshly distilled water.

Method

- Arrange the assay tubes as shown in Table XIV.
 Each standard is done in triplicate and each unknown in duplicate.
- 2. Add reagents as directed in the Table.
- After the final addition of buffer, vortex the tubes and centrifuge at 1500 g for 25 minutes.
- 4. Pour off the supernatant fluid without tapping or shaking the tubes. Allow to drain for
 15 minutes.
- 5. Gently dry the rims.
- 6. Count each sample in a gamma counter for one minute.

Calculation of Results

- Plot the count rates for each standard against the insulin concentration on linear graph paper. Draw the best curve through the mean of the triplicate points, rejecting grossly aberrant counts. (For example see Figure 19).
- 2. Using the mean of the duplicate count rates for the unknowns read off the insulin concentration from the standard curve.

								izi i			
	Total	Standards (ng/ml)				Blank	Unknown	Sample			
	Counts	0	0.4	1	2	3	4	6	1.1	1 -	N
Tube No.	1 - 3	4 - 6	7 - 9	10-12	13-15	16-18	19-21	22 -24	25-27	28-29	etc.
Buffer	-	100	-	-	-	-	-	-	200	-	-
Rat Insulin standard	-	-	100	100	100	100	100	100	-	-	-
Unknown serum	-	-	-	-	-	-	-	-	-	100	100
Binding reagent	-	-	100	100	100	100	100	100	-	100	100
Vortex. Place in refrigerator at 2-4 ⁰ C for 45 mins., then add											
125											
Insulin	100	100	100	100	100	100	100	100	100	100	100
Vortex, Place in refrigerator at 2-4 ⁰ C for 2 hours 15 mins. Then add											
Buffer	-	700	700	700	700	700	700	700	700	700	700

Vortex, centrifuge, decant and count. N.B. All volumes in microlitres

Table XIV Radioimmunoassay protocol.



Fig. 19 Radioimmunoassay of insulin. Standard curve.

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