THE IMMUNOALTERATION OF PANCREATIC ALLOGRAFTS

by

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Statement of Originality

This work is original and unless otherwise stated has been performed by myself. The work was performed at the Department of Surgery, Box 77, University of Chicago, 5841 S. Maryland, Chicago, Illinois, U.S.A. 60637.

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ABSTRACT

Since George Snell, in 1957, first postulated that transplantable tissues and organ grafts contain cells or "passenger leucocytes" which, alone, could perhaps be responsible for graft rejection and eventual graft loss, many researchers have endeavoured not only to identify these leucocytes, but have also attempted to eradicate them from the graft itself prior to transplantation. Professor Frank P.Stuart, at the University of Chicago, demonstrated that following irradiation, and subsequent elimination of these cells, animal kidneys enjoyed a prolonged survival when transplanted into non-immunosuppressed recipients. Unfortunately, many of the subsequent pretreatment regimens which were developed, including irradiation, cyclophosphamide or azothiaprine pretreatment, were only effective if performed four or five days prior to transplantation. This is clearly not applicable to the clinical situation. The removal of these passenger leucocytes or dendritic cells from transplantable tissue is fundamentally important in reducing the immunogenicity of that tissue. There is a need, therefore, to develop an effective method for removing these dendritic cells from a whole organ, prior to transplantation, which will not only be effective in reducing graft immunogenicity but also be clinically applicable.

With the increasing knowledge of what these immunogenic cells are, and with the concomitant development of monoclonal antibodies, I have been developing an animal model to study the effects of pretreating whole, intact organs with monoclonal antibodies targeted against these dendritic cells, and studying the effect upon allograft survival following transplantation after this pretreatment. An *ex vivo* perfusion circuit was developed which enabled an isolated intact rat pancreas (or kidney) to be perfused with monoclonal antibodies for up to six hours, without undue damage to the organ, and subsequently be transplanted. The effect on the survival, after transplantation, of these pancreases which had been pretreated in this way is the main issue addressed in this thesis.

The thesis is divided into three sections. Section I is the general introduction and gives an historical account of pancreas transplantation, diabetes, and previous studies dealing with tissue immunoalteration. Section II constitutes most of the experimental work surrounding the attempts at 'immune modulation'. It describes the development of the *ex vivo* perfusion circuit and reports the effects on allograft survival of transplanting pancreases perfused with anti-Class II monoclonal antibodies for different periods of time. In addition to the *ex vivo* perfusion data, this section contains the results of *in vitro* studies performed on isolated pancreatic islets. Pancreatic islets were cultured with allogeneic T lymphocytes in mixed islet lymphocyte cultures. The effect of pretreating these islets with anti-Class II MAb's on the stimulation of these T cells was studied.

Section III provides evidence that a new programme involving clinical pancreatic transplantation as a safe and reliable alternative for the treatment of diabetes can be set up successfully. The Section describes the first year's experience of pancreas transplantation at the University of Chicago.

Finally, Section III comprises the general discussion of the thesis. It concludes that the pretreatment of vascularised organ allografts can effectively reduce their immunogenicity, but not sufficiently to abrogate allograft rejection altogether. The elimination of the 'passenger' dendritic cells may be an important adjunct in the battle to reduce the severity and frequency of allograft rejection episodes, and perhaps allow reduction in non-specific immunosuppression.

This dissertation is dedicated to my mother,

Anne Lloyd

who encouraged me to pursue a medical career and
to my father,

John Alan Lloyd

whose continued support made it possible.

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SECTION I

General Introduction and Background

CHAPTER 1.

PANCREAS TRANSPLANTATION

1.1 INTRODUCTION

Sir - Can any of your readers inform me if cases of diabetes have been treated by implanting the pancreatic gland of an animal into the patient? I have at present in my wards a case of diabetes mellitus of the "bad" type and I have requested that the transplantation of pancreas shall be tried in his case..... The pancreas produces a sugar-destroying substance, which is not contained in the ordinary pancreatic secretion which flows into the duodenum, but may possibly be absorbed from the gland itself. By implanting pancreas we might get a bigger supply of this sugar destroying substance ....."

James W. Allen, M.B., C.M.,
British Medical Journal,
February 28th, 1903.

"Sir - I noticed some correspondence on a suggestion to transplant the pancreas in cases of diabetes. Why transplantation? ..... All the evidence is against the possibility of such transplanted tissue being competent to perform its normal function, even if it should persist for a time. In face of these facts I think it is quite unjustifiable to perform on a diabetic such an operation as that proposed. I am, etc.,"

Kieth Monserrat,
British Medical Journal,
March 28th, 1903.

Dr. Watson Williams at the Bristol Royal Infirmary, in 1893, transplanted some sheep's pancreas (*'three pieces each of the size of a Brazil nut'*) into the subcutaneous tissues of the abdomen and breast of a young 15 year old

boy in the pre-coma stages of diabetic ketoacidos (Williams, 1894). This first report of an attempt to treat diabetes by transplanting pancreatic tissue marks the beginning of almost a century in pancreas transplantation that has been plagued with the problems of rejection and technical failure. Needless to say that this first pancreatic 'xenograft' did not function and the boy subsequently died in diabetic coma on December 23rd, 1893.

The basis for pancreas transplantation dates back to 1889 when von Mering and Minkowski showed that pancreatectomy produced diabetes mellitus in dogs (Von Mering and Minowski, 1889). Indeed, Williams referred to their work several times during subsequent papers; but why were they considering transplantation of a gland whose function had not yet been fully established?

Diabetes was, and is, an incurable disease. A remedy for this common 'affliction' was indeed sought. Severe and debilitating complications were all too apparent in untreated patients (see Chapter 2). The isolation of insulin was not to occur until 1922, yet the treatment of diabetes by transplantation or implantation of pancreatic tissue was suggested by several investigators before then (Hedon, 1892; Minkowski, 1892; Ssobolew, 1902) and even attempted by others as early as 1893 by Williams. With the epocal studies of Banting and Best, who not only isolated and purified insulin, but also demonstrated conclusively that insulin was produced in the pancreas (Banting and Best, 1922), other workers, with renewed enthusiasm, attempted pancreas transplantation. Banting received a Nobel Prize for this work in 1923. A series of experimental animal studies were initiated, which adopted the vascular techniques of Alexis Carrel, involving whole pancreas transplantation in dogs (Gayet and Guillaumie, 1927).

Brooks and Gifford performed numerous pancreatic transplants in pancreatectomised dogs and clearly demonstrated reversal of their diabetic state (Brooks and Gifford, 1959). During the following decade many researchers, including Dejode (Dejode *et al*, 1962), Reemsta (Reemsta *et al*, 1963), Merkel (Merkel *et al*, 1967), Lillehei (Lillehei *et al*, 1967), and Largiader (Largiader *et al*, 1967), worked out the technical details in animal models.

1.2 THE EARLY EXPERIENCE OF PANCREAS TRANSPLANTATION

One seemingly insurmountable problem facing whole organ pancreas transplantation, was to determine a relatively safe method for the drainage of the exocrine secretions from the pancreatic duct. It seemed rather cumbersome to transplant the whole pancreas when only the insulin producing islets of Langerhans, making up only 1% of the total mass of the pancreas, was actually needed for metabolic homeostasis. Despite great advances techniques involving the isolation and purification of pancreatic islets, there was (and is) no efficient method available for isolating pure pancreatic islets in sufficient quantities to reverse the metabolic effects diabetes mellitus in man. Until clinical success in pancreatic islet transplantation is achieved, whole or segmental pancreas transplantation, remains a therapeutic, yet controversial, alternative.

The early clinical reports of whole pancreas transplantation clearly demonstrated that clinicians were haunted by leakage and fistulae formation from the digestive juices secreted from the pancreatic duct. A dog model for drainage of the exocrine pancreas by transplanting the duodenum along with the pancreas was described as early as 1929 (Houssay, 1929). This method was further adopted and developed by DeJode and Howard who brought the distal end of the attached duodenum as a cutaneous duodenostomy (DeJode and Howard, 1962). Largiader and colleagues later anastomosed the duodenum to the jejunum (Largiader *et al*, 1967), and clinicians were so encouraged by the apparent, although somewhat limited success of these animal procedures, that some proceeded to attempt the first human pancreas transplantation in the mid 1960s.

On December 17, 1966, at the University of Minnesota, the world's first attempt to transplant a vascularised pancreatic graft in a human was performed (Kelly and Lillehei *et al*, 1967). This was a segmental pancreas graft transplanted simultaneously with a renal graft into a 28 year old uraemic diabetic lady who was afflicted with diabetes since the age of 9. In keeping with their current research, Kelly and Lillehei elected to ligate the pancreatic duct and oversew the cut end of the pancreas. Unfortunately, the pancreas only functioned

for a few days and insulin therapy was reinstituted. The segmental graft became swollen and developed pancreatitis with accompanying fluid collections which became infected. Both the kidney and the pancreas allografts were removed after 2 months, but despite the patient recovering from the immediate surgical and pancreatic graft complications, she developed a fatal pulmonary embolus 13 days after the removal procedure.

It was unfortunate that they elected to ligate the pancreatic duct. There had been several experimental studies (Banting and Best, 1922; Ssobelow, 1902; Thiroloix, 1892) which, although had verified preservation of endocrine function after occlusion of the pancreatic duct, had demonstrated subsequent atrophy of the exocrine parenchyma. (Perhaps a welcomed consequence if the islets remained functionally intact.) However, these trials of transplanting the duct ligated dog pancreas were usually unsuccessful and were associated with severe wound complications, particularly caused by pancreatic duct fistulae. Due to a number of accessory ducts, a complete obstruction of the pancreatic ductal system is almost impossible to produce by ligation (Cross, 1956), and an incomplete obstruction is prone to cause pancreatitis and fistulation. Both Reemsta and Lillehei were aware of these problems. Reemsta attempted to eliminate the exocrine function by ductal ligation several weeks prior to transplantation (Reemsta *et al*, 1963), and Merkel, together with Lillehei, attempted reduction by graft irradiation (Merkel *et al*, 1967). Both these methods seemed to improve the results to some degree, but were not applicable to the clinical situation. It seemed suprising, therefore, that Richard Lillehei and colleagues elected to use simple duct ligation in their first patient who received a pancreas allograft.

Their second patient faired a little better. In the same year, on December 31, 1966, Lillehei and colleagues performed another pancreatic transplant (Kelly and Lillehei *et al*, 1967) in a 32 yr old Caucasian lady. On this occasion, they elected to transplant a portion of the duodenum with the *whole* pancreas, and bring the free end out as a cutaneous duodenostomy, as described by DeJode and Howard (DeJode and Howard, 1962). This patient remained insulin independent for two months when the graft function failed, presumably as a result of rejection. Of interest, was the fact that the whole length of duodenum was also successfully transplanted, which was the first report of a small bowel allograft functioning following transplantation.

In the years to follow, from 1966 to 1973, 10 uraemic diabetic patients received pancreatico-duodenal grafts at the University of Minnesota (Sutherland *et al,* 1984). The outcome in these pioneering cases was reported at the annual meeting of the American Surgical Association in 1970 (Lillehei *et al* , 1970). Only one pancreas functioned for a period of 1 year, and only one patient from this initial series was still alive in 1984. The authors commented that pancreatitis and fistulae were a major problem and alternative techniques for improved duct drainage were sought.

Many centres have now attempted pancreas transplantation such that over 1157 have been performed world wide in 76 institutions (Sutherland and Moudry, 1988). The one year pancreatic graft survival rate has increased dramatically from 3% before 1977 to 43% in 1985 (Sutherland and Moudry, International Pancreas Transplant Registry, 1987), although in some centres the allograft survival figure at one year is now 86% (Sollinger *et al,* 1987), 80% (Corry, 1988), 71% (Groth and Tyden, 1988) and 70% (Dubernard *et al,* 1988). The great improvement in the survival rates over the last decade has been achieved by a better understanding of immunosuppressive therapy, and safer techniques for controlling the exocrine secretions of the pancreas.

1.3 SURGICAL TECHNIQUES FOR PANCREATIC DUCT MANAGEMENT

1.3.1 Enteric Diversion of the Pancreatic Exocrine Secretions

It seemed only logical to divert the pancreatic juices into the gastrointestinal tract. Pancreatico-enterostomy offers the most physiological way to handle the exocrine secretions. There have been several extensive (and ingenious) surgical techniques developed to attempt safe enteric drainage for both the segmental graft and the whole pancreas graft with or without its accompanying duodenum. Both Groth and Sutherland have reported extensively on the variations of anastomosing segmental grafts to the recipient's bowel.

Groth and colleagues (Groth *et al.*, 1976) used an end-to-end pancreatico-Roux-en-Y loop in their first five cases. In many of these patients a pancreatic fistula developed. They changed to ducto-enterostomy using either a Roux-en-Y loop or simple ileal U-loop. Again there were fistulous complications (Groth *et al*, 1984). Sutherland *et al.* were experiencing similar problems with Roux-en-Y loops sleeved over the cut end of the pancreas (Roux-en-Y pancreatico-jejunostomy) and with anastomosis of the bowel to the pancreatic duct itself, pancreaticodocho-jejunostomy (Sutherland *et al*, 1984). In their initial series of one hundred pancreas transplants performed at a single institution, 29 were transplanted with exocrine drainage into the gut. Eight failed due to technical reasons with fistulae, abscesses and severe wound infections.

The results of segmental transplantation with enteric drainage were discouraging. Whole pancreas transplantation with enteric drainage did not fair any better. Draining the pancreatic juices into the bowel meant that the proenzymes became activated and consequently the anastomosis was exposed to highly active digestive enzymes. Enterically drained pancreases were inevitably accompanied by a high incidence of anastomotic leaks perhaps for this reason. The pancreatic fistulae that developed invariably became infected and would not heal. Consequently, many of the grafts had to be removed.

Recently, however, some technical advances have led to a significant reduction in the incidence of these complications. In whole organ transplantation, the revival of the duodenal segment as a conduit for the exocrine secretion has reduced the leakage rate and proven advantageous (suggested by Starzl *et al,* 1984). This is particularly applicable to urinary diversion (see below) but has lowered complications in enteric diversion too. In the case of small bowel drained segmental grafting, the use of a pancreatic duct catheter to temporarily divert the exocrine secretions to the exterior for the first few weeks after transplantation has been found to be effective in preventing anastomotic leakage (Groth and Tyden, 1988). Overall, the results recently reported to the Pancreas Transplant Registry do not reflect any significant difference in patient or graft survival with enterically drained or bladder drained techniques. However, many of the new centres beginning pancreas transplantation, particularly in the States, are adopting alternative methods for dealing with the exocrine secretions.

1.3.2 Pancreatic Duct Injection

Pancreatic duct occlusion by injection of a synthetic polymer seemed to be one alternative. From Europe, in 1978, Dubernard *et al.* reported on the use of neoprene for the total obliteration of the ductal system in dogs and suggested its application in segmental pancreas transplantation (Dubernard *et al,* 1978). This technique had also been applied in general surgery in cases of pancreatic resection (Di Carlo *et al,* 1984), as it is technically simple and relatively safe. *In situ* duct occlusion with neoprene was shown to stop the exocrine pancreatic secretions and produce a complete atrophy of the exocrine tissue, apparently without affecting the endocrine pancreas (Dubernard *et al,* 1979). Similar results had previously been reported by Anylan and colleagues, who observed normal glucose tolerance tests in dogs 6 months after duct occlusion with vinyl plastic (Anylan *et al,* 1957). Subsequently, other obliterating substances such as acrylate glue (Little *et al,* 1977), prolamine (Gebhardt and Stolte, 1978), polyisoprene (McMaster *et al,* 1981) and silicone rubber compounds (White *et al,* 1981) were shown to give equivalent results.

The duct occlusion technique was adopted in clinical pancreas transplantation. It had a low procedure-related complication rate and soon became the most frequently applied method for handling the exocrine pancreas, and remains particularly popular in Europe. This technique has been, and still is, greatly advocated by Dubernard.

However, it has been suggested that ductal occlusion interfered with the normal endocrine pancreatic function, and incidents of graft failure were attributed to fibrotic degeneration of the acinar tissue. The fibrosis was assumed to interfere with the blood supply to the islets. In dogs, however, no reduction in pancreatic blood flow was found in the course of 8 months after duct occlusion (Gooszen *et al,* 1983). One study in pigs, on the other hand, showed a significantly reduced pancreatic blood flow 6 weeks after *in situ* duct occlusion with prolamine (Wayand *et al,* 1982), even though normal glucose tolerance was retained in these animals.

Numerous extensive light microscopic studies have shown the fate of the exocrine pancreatic tissue after duct obliteration (Gebhardt and Stolte, 1978; Gooszen *et al,* 1984). Duct ligation, when complete, and ductal occlusion by

obliterative injection, produce principally identical changes in various animal models (Orloff *et al*, 1975; Pound and Walker, 1981). During 1- 3 months following the procedures, a total atrophy of acinar tissue occurs which is replaced by dilated ductules and interstitial connective tissue. Later on, the ductules and connective tissue are gradually replaced by more or less dense fibrous tissue. During this time, the islets show a remarkable spectrum of morphological changes. Fairbrother *et al*, 1980, reported that fragmentation and scarcity of islets is a common sequalae, and similar findings have been reported by others (Gooszen *et al*, 1984). However, Dubernard believes that although there is significant destruction of the exocrine tissue, the majority of the pancreatic islets are preserved. He suggests that the islet destruction reported in the animal studies is due a combination of other factors such as preservation injury, surgical insult or perhaps a consequence of high dose immunosuppression (cyclosporine) and that the fibrosis, in his experience, is limited only to the acinar tissue (Dubernard *et al*, 1979). Furthermore, in the recent International Congress of the Transplantation Society, held in Sydney, Australia, 1988, he claimed that a vascularised segmental pancreatic allograft with ductal obliteration is the only method available to transplant a functioning 'islet' allograft (personal communication). He suggests that the exocrine tissue becomes almost completely destroyed and the insulin-producing islets remain the only functioning part of the vascularised graft. His current one year pancreatic allograft survival of 71%, with neoprene injection, is similar to other current graft survival figures reported using alternative methods for ductal drainage (Dubernard *et al*, 1988).

1.3.3 Urinary Diversion of the Exocrine Secretions

The quest for finding the safest surgical procedure for dealing with the notorious duct secretions continues, although, perhaps we are now reaching that goal. In 1973, Gleidman reported their experience with five patients who underwent pancreas transplantation with ureter-pancreatic duct anastomosis for exocrine drainage (Gleidman *et al*, 1973). Two of these patients had functioning allografts for almost one year. MacDonald and colleagues later reported success

with this technique in animals (MacDonald *et al,* 1982). Though Starzl introduced the concept of the duodenal segment as a conduit for aiding pancreatic exocrine drainage (Starzl *et al,* 1984), it is to Sollinger that the major credit for the development of urinary tract drainage should be given (Sollinger *et al,* 1985).

In 1983, Sollinger and Cook demonstrated that pancreatic duct to bladder anastomosis was a safe and a reliable procedure in dogs (Cook *et al,* 1983). They were able to demonstrate that with the use of cyclosporine, the animals had functioning allografts for several months and that there was no detectable damage to the bladder epithelium and that metabolic equilibrium was maintained. They performed their first human segmental pancreas transplant utilising this novel technique in June 1982 (Sollinger and Belzer, 1988). Since then, they have performed over 65 cases with more than 100 cases being performed world wide using a bladder drainage procedure. Between June 1982 and April 1986 a total of 23 institutions have performed pancreas transplants using this (or a slight modification of this) technique.

The original description by Cook involved segmental grafts directly anastomosed to the bladder, with the vascular pedicle attached to the iliac vessels. However, in the initial clinical series, there were several cases of vascular thrombosis, despite the use of a distal A-V fistula as advocated by Calne (Calne *et al,* 1980). Consequently, the segmental technique was abandoned and whole grafts were transplanted (Sollinger *et al,* 1985). The use of the duodenal 'button' was thus described for the first time. Prior to transplantation, the duodenum was separated from the pancreatic uncinate process except for a small 'button' of duodenum measuring 5-6 cm surrounding the ampulla of Vater. The immediate improvements of graft survival were apparent, but the technique was rather involved and required extra time and concentration in preparing the button for anastomosis to the bladder at the end of, what was usually, a long and protracted harvesting procedure. The technique was to be modified even further.

The improved results were enthusiastically greeted by Corry at the University of Iowa. Although Corry attempted the duodenal 'button' procedure, it was he and Nghiem who introduced the short duodenal segment as a conduit between the donor pancreas and the recipient bladder (Ngheim *et al,* 1986). Indeed, the one year graft survival figure is 80% (Corry, 1988). It is this particular technique which is now proving successful throughout the world, and

is even being adopted by both Sutherland and Sollinger. Certainly, many other centres are now using this technique, as it is relatively simple and carries an extremely low incidence of surgical complications. It is this technique, utilising a doudenal conduit, which has been introduced at the University of Chicago, the results of which will be described in Chapter 10.

1.4 INSULIN DELIVERY INTO THE PORTAL CIRCULATION

The techniques employed to minimise complications from the exocrine pancreas have usually been associated with systemic drainage of the insulin from the pancreatic graft. It would be more physiological to attempt drainage into the recipient's portal circulation. Indeed Calne has advocated this procedure and reported "paratopic" allografting of the pancreas (Calne, 1984). In his series, the pancreatic segments were anastomosed to the recipient's own splenic vessels and the pancreatic ducts drained into the stomach. Furthermore, he has reported that the activation of the pancreatic enzymes was inhibited in the stomach because of the low pH (Calne, 1985). He also advocates use of a distal arteriovenous fistula on the graft splenic vessels to enhance blood flow and reduce the rate of venous thrombosis in the donor splenic vein (Calne *et al,* 1980). The initial results are encouraging with a reported actuarial graft survival rate of 50% (Calne and Brons, 1988).

In an attempt to achieve splanchnic drainage of insulin, others have anastomosed the graft portal vein to the superior mesenteric vein (Tyden *et al,* 1985) or even the inferior mesenteric vein (Sutherland *et al,* 1987) of the recipient. However, the advantages for the portal drainage of insulin are at present theoretical, and the high incidence of surgical complications associated with the increased difficulty utilising this technique has made it less attractive.

The systemic drainage of insulin results in many patients having raised C-peptide levels and having hyperinsulinaemia, which is also exacerbated by the reduced insulin sensitivity caused by corticosteroid medication. The long term consequences are unknown, but some authors suggest a possible correlation

between hyperinsulinaemia and macroangiopathy (Weatherall *et al,* 1987).

1.5 THE EFFECT OF PANCREAS TRANSPLANTATION ON DIABETIC COMPLICATIONS

The prime goal for the pancreas transplant is to provide a normal metabolic control and prevent the progression of the devastating complications of diabetes mellitus. The transplant undoubtedly improves the patient's quality of life, but if the complications of diabetes are not affected, then the patient merely exchanges the complications of insulin therapy for those of life long immunosuppression (see Chapter 2). Many centres overcome this dilemma by electing to transplant diabetic patients who are in renal failure secondary to diabetes, and require both a kidney and pancreas transplant. Indeed, 25-30 % of patients on many renal transplant waiting lists are diabetic. These patients would receive a kidney allograft with it's concomitant immunosuppression and could potentially receive a pancreas transplant from the same donor at the same time. The patients would be given the pancreas transplant almost as a bonus since they would already be receiving long term immunosuppression because of their renal allograft.

It must also be mentioned that diabetes, unlike liver failure or heart failure, is not an acutely fatal disease. The pancreas transplant procedure must be safe enough to have zero mortality associated with it. Only then can transplant surgeons justify performing this procedure with the hope that the reduction in the morbidity from diabetes far outweighs the morbidity (and mortality) of the surgical procedure.

If the purpose of pancreas transplantation is to halt or even reverse the complications of diabetes, then whether this can be achieved by a normalisation of the metabolic abnormalities of diabetes remains in debate. There are several conflicting reports in animals.

Orloff demonstrated conclusively that in an animal model, the diabetic complications could not only be prevented by pancreatic transplantation, but that

even established diabetic renal lesions may be restored to normal. Furthermore, when the transplant is performed as late as two years after induction of diabetes, there was significant improvement in the diabetic complications (Orloff *et al,* 1986).

In contrast to this, in man, the effect of strict metabolic control, as achieved by multiple insulin injections or subcutaneous insulin delivery, remains equivocal. A progression in diabetic retinopathy was reported in patients despite close monitoring and intensified treatment for 1 year, but 2 years after treatment some improvements were noted in background retinopathy (Kroc Collaborative Study Group, 1985; Lauritzen *et al,* 1985; Dahl-Jorgensen *et al,* 1986). Furthermore, Feldt-Rasmusen *et al,* reported that there was no further progression in early diabetic nephropathy after 2 years of strict metabolic control (Feldt-Rasmusen *et al,* 1986). This was also noted elsewhere (Dahl-Jorgensen *et al,* 1986).

The effects of pancreatic allografting on diabetic retinopathy are disappointing. There are reports that the retinopathy is improved following transplantation (Landgraf *et al,* 1987). Indeed, in those cases tested, 10 out of 16 patients had improvement in their ocular symptoms, and the visual acuity improved dramatically in one. The recent report by the Minnesota group, however, reflects the pessimistic results that were expected (Ramsay *et al,* 1988). They concluded that the progression of diabetic retinopathy continued despite pancreas transplantation, and that there was no discernible difference in the deterioration between those patients receiving a pancreas transplant and those who did not. (Ramsay *et al,* 1988). These disappointing results have also been corroborated by others (Wilczek *et al,* 1987).

Peripheral neuropathy is a common and almost obligatory sequelae to diabetes. Subjective signs include paraesthesia, muscle weakness and leg cramps. Autonomic dysfunction presents as constipation, hypoacidity, gastroparesis, impotence and orthostatic hypotension. Measurements of nerve conduction velocities give a more objective view. Indeed, Landgraf and colleagues reported moderate improvements in the nerve conduction velocities of the median, peroneal and sural nerves following pancreas transplantation

(Landgraf *et al*, 1986). This is in contrast to the lack of improvements on the diabetic complications in the eye. In all the patients tested, there was a dramatic improvement in nerve conduction velocities, sympathetic responses, and thermographic characteristics in the leg. These findings have also been recently confirmed by others (Wilzek *et al*, 1987). However, most of the patients had all undergone combined pancreatic-renal transplantation and the improvement could have been attributed to the cure of uraemia, as similar results have been reported in diabetic patients receiving a kidney allograft alone.

Sutherland *et al* have some evidence that neuropathic complications are improved by pancreatic transplantation (Sutherland *et al,* 1986). They confirmed that in diabetic patients who received only a pancreas transplant, there was a general improvement in both sensory and motor condition at one year post transplant. Furthermore, they concluded that the overall neurological status of the recipients improved when all parameters including autonomic and clinical conditions, were taken into account.

The effect of pancreas transplantation on the autonomic neuropathy has primarily been addressed by studies on the cardiovascular disturbances. Thus, the relative beat to beat variation of the ECG during deep breathing has been used to assess the parasympathetic vagal reflex arc, and the increase in heart rate following orthostatic testing to study the sympathetic part. Schafferhans were not able to demonstrate improvement in the parasympathetic system, although Sutherland's group demonstrated that the beat to beat variation either improved or stabilised after transplantation. Wilczek and colleagues, also showed an improvement in sympathetic responses but normal values were not attained (Wilczek *et al*, 1987).

1.6 CONCLUSION

Experience with clinical pancreas transplantation has almost spanned a century and until these past few years transplant surgeons (and patients) have been plagued by the complications of the procedure. It is really in the past two or

three years that some programmes are reporting 70% or 80% pancreatic allograft survival at one year, but the cumulative world data, taken from the Pancreas Transplant Registry, reflects the abysmal results from other centres as well. It seems that techniques involving segmental grafts or enterically drained grafts, whether whole or segmental, have an increased complication rate, particularly caused by leakage from the pancreatic duct. In the case of an enterically drained pancreas, there is the additional complication of pancreatic enzymic activation.

In the light of the world experience, it seems that a whole pancreas graft, with a duodenal conduit anastomosed to the bladder for exocrine drainage, offers the safest procedure. Furthermore, rejection of the pancreas can be detected by a fall in urinary amylase levels and also, perhaps, by an analysis of the cellular components in the pancreatic duct fluid.

There still remains great contoversy regarding the ethical and scientific recommendations for pancreatic transplantation in Type 1 diabetics (Pyke, 1988). Until a well constructed, multicentre, prospective, randomised control trial is instituted, then the debate will continue *ad infinitum*. It must be verified whether the progression of the diabetic complications can be stopped or reversed by this procedure. Except for research, which must continue in a few specialised centres, it seems unjustifiable to perform a pancreatic transplant in the newly diagnosed diabetic with little or no nephropathy, because the risks of immunosuppression are too high. However, pancreatic transplantation, combined with renal transplantation, would offer the patient a much improved quality of life, and protect the kidney from graft diabetic nephropathy.

CHAPTER 2

DIABETES

2.1 INTRODUCTION - THE HISTORY OF DIABETES

Diabetes is a disease of antiquity. A treatment for polyuria is given in the first reference to diabetes in the Papyrus Ebers (Plate 2.1), an ancient text, dating back to 1500 B.C. (Ebbell, 1937), but the first description of the disease is attributed to Aretaeus, the Cappadocian, between A.D. 30 - 90 (Adams, 1856; Wrenshall *et al,* 1962). He gave a wonderful account of the disease and probably was the first to use the term 'diabetes', from the Greek 'διαβητης' meaning 'a syphon' (Leopold, 1930; Adams, 1856). Hippocrates (469 - 399 B.C.) and Aristotle's (384 - 322 B.C.) comments on polyuria may have also alluded to the disease, while Celcius (30 B.C. - 50 A.D.) gives an impressive, albeit erroneous, description (Gemmill, 1972). Polyuria was recognised in China in the third century A.D., but sweet urine *("so sweet as to attract dogs")* was not recorded until the seventh century (Allen, 1932). Sustruta, an Indian physician of the fifth century, named the disease "Madhumeha" or "Honey Urine". The Arabian physician, Avicenna (980 - 1027 A.D.) first described the complications of diabetes - carbuncles, phthisis and gangrene (Barach, 1928), while osteitis, another complication, may have caused the erosions found in ancient skeletal remains. Thomas Willis (1621-1675) realised the importance of glycosuria -*"the urine...was wonderfully sweet as it were imbued with Honey or Sugar"* and his papers were published soon after his death (Willis, 1679).

It was not until Oskar Minkowski (Plate 2.2), in 1889, following an arguement with his colleague, von Mering, over whether or not an animal could survive total removal of the pancreas, that led him to perform total pancreatectomies in dogs (von Mering and Minkowski, 1892). The association of diabetes with the pancreas gland was firmly established. Previously, J.C. Brunner (1653 - 1727) performed total pancreatectomy in dogs and noted

PLATE 2.1

Plate 2.1 Photograph of an extract from the *Papyrus Ebers* (1500 B.C.)

polydipsia and polyuria, but he failed to recocognise diabetes (Labhart, 1978). With increasing surgical interest surrounding the pancreas, Laguesse suggested that diabetes was prevented by some form of internal secretion, perhaps from small clusters of cells within the pancreas originally described by Paul Langerhans in 1869 (Laguesse, 1893). Laguesse called them "Islets of Langerhans". That these specialised groups of cells were indeed responsible for the 'internal secretion' was shown by Ssobelow, who showed that duct ligation caused atrophy of the exocrine tissue but left the islets intact (Ssobelow, 1902). The animals did not develop diabetes until that remaining portion of the pancreas was ultimately removed.

Insulin was finally isolated in 1922 by Banting and Best. They injected their extract from a calf's pancreas into a diabetic patient and reported the results (Banting and Best, 1922). Since then, more purified insulins have been isolated, and repeated subcutaneous insulin therapy has become the standard treatment for diabetes. However, despite intense medical supervision and extremely careful metabolic control, diabetics are still at risk from the devastating and debilitating complications of this disease.

2.2 AETIOLOGY

The cause of insulin-dependent diabetes mellitus (IDDM or Type - 1) is unknown. It is associated with HLA antigens, particularly DR3 and DR4, expressed on the surface of particular leucocytes in the blood. Other combinations may distinguish people at high risk, particularly C4-B2 or C4-B4, and the expression of B8-B15 has recently been implicated. DR3 and DR4 antigens can be demonstrated in 95% of the patients with IDDM, whereas they are present in only 50% of the background population. However, for the development of the disease, the presence of these two HLA markers is not sufficient. Only 25 - 50 % of monozygotic twins will become concordant in respect to diabetes, which means that not only must there be a genetic predisposition, but that environmental factors must also be responsible. These may include certain viruses, perhaps mumps or Coxsackie B_4, or chemical compounds such as nitrosamines. Circulating islet cell antibodies can be

PLATE 2.2

Courtesy of "Münchener Medizinische Wochenschrift"

OSKAR MINKOWSKI (1858-1931)

Plate 2.2 A photograph of Oskar Minkowski who demonstrated in 1889, that removal of the pancreas resulted in diabetes mellitus.

demonstrated in many patients, and a lymphocytic infiltration can be found surrounding the islets of Langerhans. It can be postulated that the increased expression of these MHC antigens in diabetics leads to activation of T cells which specifically destroy the β-cells within the pancreas.

2.3 PATHOLOGY AND COMPLICATIONS OF DIABETES

Diabetes mellitus is a syndrome characterised by insufficient insulin secretion, reduced glucose tolerance and development of universal microangiopathy, neuropathy and arteriosclerosis. Amoung Western countries the prevalence of diabetes is about 2%, but the frequency of the disease increases with age such that almost 10% of the population die with diabetes, but only 1.5% will die of diabetes. In the U.K. the incidence is 2 per 1000 population, with peaks at 5 years and adolescence. There is an increased incidence in Scandinavians and diabetes is more uncommon in Asians.

Diabetes mellitus reduces the quality as well as the duration of life due to the effects on the heart, kidney, eyes, arteries, brain, and legs. The nervous system is also affected. Coronary heart disease and uraemia are the most frequent causes of death among diabetic patients.

Due to better access to medical care, less than 20% of patients today have ketoacidosis when diabetes is diagnosed. However, glycosylated haemoglobin, Hb A_{1c}, a marker raised in patients with poor glucose control, is usually extremely high. After initial insulin therapy the patient becomes symtomless and after several weeks about 25% of patients will undergo a remission period. During this time the insulin requirements can be quite low due to recovery of endogenous insulin secretion. After about six months the remission period is over, blood glucose will fluctuate and diabetes can be difficult to treat. Two or three insulin injections a day may be necessary to control the blood sugar, but the Hb A_{1c} often remains abnormally high. If the problems are severe, insulin infusion pumps might be of benefit. Height development in children might be impaired, so too is the development of the fetus in pregnant women, who have increased risk of polyhydramnios and toxicosis.

FIG. 2.1

COMPLICATIONS OF DIABETES

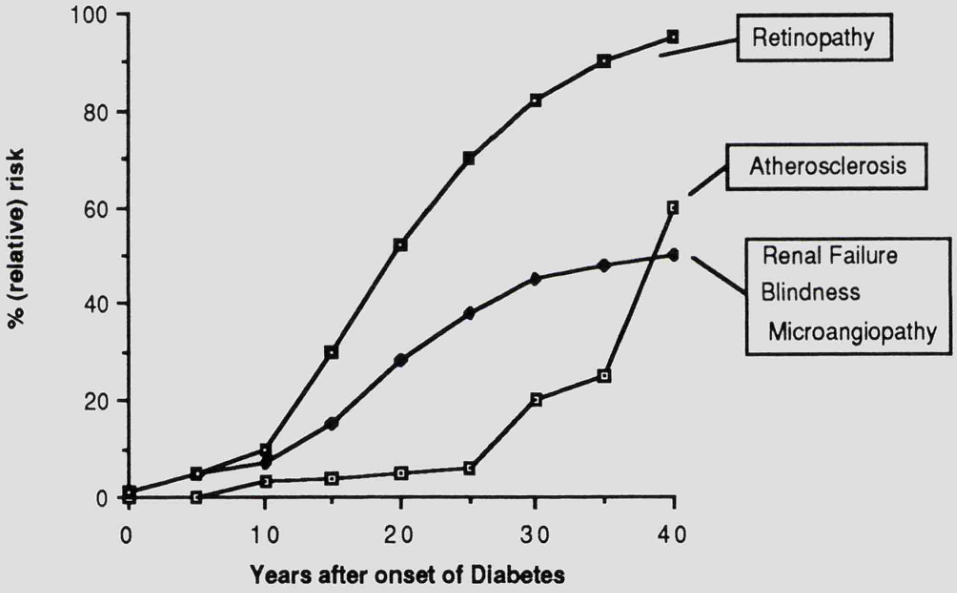

Fig. 2.1 Complications of Diabetes Mellitus. This graph shows the increase in incidence of severe diabetic complications with duration of disease. Almost 100% of patients will develop retinopathy, 40% will become blind in untreated cases, 40% will develop renal failure, and 60% will develop severe atherosclerosis 30 years after the onset of the disease.

Overall, the relative mortality has decreased about 30%, but compared to age- and sex-matched non-diabetics, the mortality of insulin-dependent diabetic patients who develop the disease before the age of 31 is significantly high, with the highest relative mortality in patients who developed the disease before the age of 10 years. The late diabetic complications are organ lesions primarily are due to microangiopathy. However, large vessel disease (macroangiopathy) contributes significantly to the mortality and morbidity also. It is characterised as atherosclerosis with intimal proliferation and narrowing of the vessel lumen. In diabetics, the disease is even more widespread, involving arteries further toward the periphery compared with non-diabetic atherosclerotic patients. The overall incidence of cardiovascular complications in patients with Type 1 diabetes is significantly higher compared to non-diabetic controls. Relative mortality from coronary heart disease is 10 times higher in patients with associated renal failure and 4 times higher in patients without clinical nephropathy. Furthermore, many risk factors associated with myocardial infarction, have been shown to be increased in the diabetic, including blood pressure, cholesterol, fibrinogen, platelet adhesion and even cigarette smoking. Death from coronary vessel occlusion is about 2 or 3 times as likely in these patients. Similarly, cerebral stroke is three times as frequent in diabetics compared to non-diabetic subjects and death rates are higher also. In the legs, the large vessel disease causes intermittent claudication, rest pain, ischaemic foot ulcers and gangrene, often necessitating amputation. The prevalence of amputation is 20 times higher in the diabetic as compared to the non-diabetic.

Microangiopathy is present in almost 100% of diabetic patients, the severity of which depends on the duration of the disease. The thickened basement membrane of capillaries effects small vessels in the kidney and retina particularly. In fact, all patients with moderate or poor diabetic control will develop basement membrane thickening, microaneurysms in the retina and glomerulosclerosis in the kidney.

The effects on the eye can be devastating. After 5 to 10 years microaneurysms will develop in the retina with the appearance of small haemorrhages and hard exudates. After 15 or 20 years, background retinopathy is present in almost 100% of cases (See Fig. 2.1). These changes will not necessarily lead to major disturbances in vision although almost half of the patients will have impaired eyesight. However, the retinopathy usually

TABLE 2.1

THE CHRONIC COMPLICATIONS OF DIABETES

Complication	Possible Cause
Macroangiopathy (*including atherosclerosis, and myocardial disease)*	Hyperlipidaemia Hyperinsulinaemia ? hyperglycaemia ? increased growth hormone ? platelets
Microangiopathy (*including retinopathy, nephropathy, capillary basement membrane thickening*)	Hyperglycaemia Protein glycosylation Hormonal factors e.g. G.H. Insulin deficiency
Neuropathy	Sorbitol accumulation Deficient myoinositol Myelin glycosylation Hyperglycaemia
Diabetic Cataract	Hyperglycaemia Sorbitol accumulation Protein glycosylation
Collagen Change	Glycosylation

From *Oxford Texbook of Medicine*, Weatherall *et al*, 2nd Ed., 1987.

Table 2.1 A list of some of the more important complications of diabetes, with a list of possible biochemical causes.

progresses to a proliferative type which is characterised by soft cotton wool exudates, major retinal haemorrhages and infarcts. Only five years after the onset of proliferative retinopathy 50% of untreated patients will become blind in both eyes. Furthermore, since cataracts, glaucoma and optimal neuritis are more frequent in the diabetic than in the non-diabetic, diabetes is the most common cause of acquired blindness in persons under the age of 60 yrs.

The effects on the kidney can almost be as devastating. Diabetes can cause many types of renal damage including diffuse or nodular glomerulosclerosis, arteriolar hyalinisation, tubular damage and exudative lesions with fibrin caps and capsular drops. Clinical diabetic nephropathy is characterised by persistent proteinuria, decreasing glomerular filtration rate and increasing blood pressure.

The glomerular lesions are due to accumulation of basement membrane-like material in the mesangium. Clinical nephropathy will develop in about 40 % of patients such that in most renal transplant centres, renal failure due to diabetes accounts for 25 - 30% of patients awaiting kidney transplant. Moreover, seven years after the onset of persistent proteinuria, 50% of untreated patients have died. Microalbuminuria is a good predictor of persistent proteinuria such that the interval to complete renal failure (and therefore dialysis or transplantation) can be estimated in the majority of cases.

Diabetic neuropathy (polyneuropathy) is characterised by degenerations of the axon and segmental demyelinisation. Almost 100% of patients will develop some aspect of polyneuropathy which becomes progressively debilitating with age. The symptoms include paraesthesia, pain and occasional paresis. Autonomic neuronal disturbances affect the gastrointestinal tract, with disturbances of gastric emptying and nocturnal diarrhoea. Difficulties in urinating, orthostatic hypotension, changes in cardiac beat to beat variation and impotence are all characteristic.

2.4 CONCLUSION

Diabetes is a severe debilitating disease. Over one half of diabetic patients will develop serious target organ complications and almost all of them will have retinopathy. Approximately 80% will die from the cardiovascular sequelae of their

disease. Even though there has been an improvement in both mortality and morbidity, due to better patient care, improved glucose monitoring and better delivery of exogenous insulin, there is still an obvious necessity to improve treatment modalities in an attempt to prevent the progression of the complications of diabetes. Thus, the rationale for pancreas transplantation is to prevent the establishment of these systemic complications, by the 'physiologic control' of the euglycaemic state.

CHAPTER 3

THE PRETREATMENT OF TRANSPLANTABLE TISSUES

3.1 INTRODUCTION

Since the early experience in whole organ transplantation the inevitable consequences of allograft rejection have been apparent. The mandatory use of potent immunosuppressive drugs has fortunately overcome most of the rejection episodes. Cyclosporine, introduced in the early 1980's, has contributed significantly to the improvement in allograft survival of all organs transplanted in recent years. However, the use of these immunosuppressive agents has not been without toll. Numerous side effects preclude their use in many cases, and the increasing incidence of infection and malignant change in those patients receiving the drugs (Table 3.1) are responsible for both increased morbidity and mortality.

George Snell postulated that their may be certain haemopoeitic agents within an organ allograft which, if removed, could reduce the severity of the rejection episodes (Snell, 1957). An understanding of the basic mechanisms underlying allograft rejection is essential if scientific methods are to overcome their effects. Furthermore, if the pretreatment of tissues can effectively reduce their immunogenicity, then perhaps application to a vascularised whole graft could be achieved.

3.2 REJECTION AND REJECTION OF THE PANCREAS

3.2.1 Historical Perspective

In 1902, at the Vienna Medical School, Emerick Ullman performed the first experimental kidney transplant in the dog, and caused considerable comment when he read his paper to the Vienna Medical Society on March 1st, 1902. He performed several autografts and xenografts from dogs to goats, but did not

continue this work for long. In France, in the Department of Surgery at the University of Lyon, Mathieu Jaboulay (1860 - 1913) headed a research programme involved with the techniques of vascular anastomosis, assisted by Carrel, Briau and Villard. Jaboulay claimed he was the first to perform a human xenograft kidney transplant (Jaboulay, 1906) but was preceded by Ullmann. Alexis Carrel left Jaboulay's laboratory and emigrated to America where he further developed methods of blood vessel anastomosis. In 1912, having spent some time at the University of Chicago, Alexis Carrel was awarded a Nobel Prize for his original and revolutionary work on vascular anastomosis. Both he and Ullman, therefore, were amoung the first to study the transferring of organs in animals. Furthermore, Carrel recognized that transplanted cat kidneys functioned for a while but some *'phenomenon'* other than classical infarction or infection caused their loss.

> *"The surgical side of the transplantation of organs is now completed.....our efforts must now be directed toward the biological methods which will prevent the reaction of the organism against foreign tissue and allow the adapting of homoplastic grafts to their hosts."*

The realisation that the loss of the grafted organ was due to a process quite distinct from loss of blood supply, infection or inflammation lead to the term *rejection* which indicated a process by which the new host denied the organ a new place of residence. The work of Medawar and his colleagues set the stage for our understanding of the pathology of rejection; they demonstrated beyond any doubt that allograft destruction is a specific immune response provoked by antigenic components in the foreign tissue (Gibson and Medawar, 1943).

3.2.2 Rejection of the Pancreas

Since the early experience in pancreas transplantation, there has been speculation whether the pancreas is indeed more susceptible to rejection than other whole organs (Lillehei *et al,* 1971). Lillehei reported on a patient who had a combined kidney and pancreatic allograft and 10 months following

transplantation, discontinued all immunosuppressive drugs for 4 days. The renal graft was lost through vigorous rejection but suprisingly the pancreas remained and the blood sugar controlled with normal levels. He also reported several cases where renal graft episodes occured with no concomitant signs of pancreatic graft rejection. Lillehei proposed that the pancreas was less prone to rejection than the kidney and that the pancreas was the least antigenic organ. Similar observations have been made by other authors including Dubernard *et al,* (1980), Baumgartner *et al,* (1983) and Tyden *et al,* (1984, 1986). In one case, the renal allograft was lost and, despite showing high levels of circulating cytotoxic antibodies against donor cells, the pancreas continued to display excellent function. These observations strongly suggest that the pancreas is *less* prone to rejection than the kidney following combined renal-pancreas allo-transplantation. It may be that pancreas graft rejection is less easily detected by current methods and that we are not recognising it early enough.

In recipients of pancreatic grafts alone, i.e. non-uraemic patients, or in patients with a previous renal allograft from a different donor, the case is quite different. In the early Stockholm report of nine technically successful pancreatic allografts, all were lost in acute irreversible rejection (Groth *et al,* 1980; Tyden *et al,* 1984). That pancreatic rejection constitutes a major cause of graft loss has been confirmed by Dubernard (1980) and Prieto (1987). Indeed, in the recent Minnesota series consisting of nearly 100 single pancreas transplants, acute rejection was the most common cause of graft loss (Sutherland *et al,* 1984). As the incidence of technical failures following pancreatic transplantation are reduced, difficulties with diagnosis and treatment of rejection will become the major stumbling block towards success. It is not that the pancreas is more prone to rejection than other organs. On the contrary, it seems that the pancreas may be less antigenic than perhaps the kidney, but efficient ways of detecting rejection episodes early enough elude us for the time being.

Schulak and Drevyanko determined in the rat the earliest histological signs of pancreatic graft rejection by biopsying allografts daily following transplantation (Schulak and Drevyanko, 1985). Histological evidence of rejection of the exocrine pancreas occured earlier than that of endocrine tissue. Indeed, by day three, a perivascular lymphoid infiltrate was found in the interstitium, while the pancreatic islets were relatively normal. Similarly, Severyn and colleagues reported on biopsy findings in dogs subjected to combined

pancreas and renal allografts (Severyn *et al,* 1982). At the onset of kidney rejection, but before functional impairment of the pancreas was noted, renal graft biopsies revealed generalised mononuclear infiltration, while biopsies of the pancreas showed diffuse interstitial infiltration, but the islets of Langerhans appeared to be spared. It seems there is a preferential cellular destruction of the acinar tissue compared to islets. Also, Groth reported clinical histological evidence of perivascular infiltration of acinar tissue by lymphocytes and granulocytes in a pancreatic allograft that was removed 2 days after cessation of function; despite some cellular degeneration within the islets, there was little or no cellular infiltration (Groth *et al,* 1976). It seems that during pancreatic graft rejection the exocrine part of the gland is involved earlier than the endocrine portion, and that the latter is afflicted at a later and more irreversible stage in the rejection process.

The clinical signs of pancreas allograft rejection have proven limiting. By the time conventional signs, i.e. hyperglycaemia, have indicated graft destruction (rejection), more than 95% of the endocrine tissue has been destroyed. Recovery from this extensive cellular infiltrative destruction is unlikely and has certainly proven difficult. General clinical signs of allograft rejection, such as fever, may occur but are inconsistent, and also occur during other entities such as inflammation and infection. Serum amylase has not proved to be reliable. A raised serum amylase level could be expected to serve as an indicator of injury to the exocrine pancreas, but this would only occur when the exocrine function of the graft had been preserved. It would not be helpful in duct obliterated grafts, for example. However, in the Minnesota experience, *serum* amylase has not been a useful predictor or indicator of graft rejection (Sutherland *et al,* 1984). If the rejecting process affects the exocrine portion of the pancreas first, it would seem logical to assume that the amylase levels in the pancreatic juice would decline during this destruction. Indeed, clearcut reductions in the amylase excretion from a pancreatic graft were first noted during rejection episodes in dogs with pancreatico-cystostomy (Gotoh *et al,* 1984). Others soon reported a marked drop in the *urinary* amylase levels during graft rejection, in patients following combined renal and pancreas transplantation with both organs draining their secretions into the bladder (Sollinger *et al,* 1984; Gil-Vernet *et al,* 1985). This has also been confirmed by the Minnesota group (Prieto *et al,* 1987). Many centres are adopting the technique of draining the pancreatic exocrine

secretions into the urinary bladder and use urinary amylase as the prime marker for pancreatic rejection, particularly following single pancreatic transplantation. However, others have not seen such an excellent correlation between declining levels of urinary amylase and early graft destruction. At the University of Chicago, two of the first eleven patients who underwent combined renal and pancreas allografts had episodes of biopsy-proven rejection in the kidney, but did not demonstrate a significant fall in their urinary amylase levels (Thistlethwaite, 1988). Some difficulties might also exist with the interpretation of the amylase data during great variations in urinary output (Munda *et al,* 1985).

A recent technique to aid the diagnosis of early pancreatic graft rejection has been postulated by Tydén *et al,* 1987. He advocated the use of pancreatic juice cytology. During early cellular rejection, lymphoblasts invade the gland and are shed into the pancreatic duct. If the pancreatic juice is diverted to the exterior daily determinations of pancreas juice cytology can be performed. Tydén has shown this to be more sensitive than current other methods. However, the contaminating cellular debris of inflammation during the first few days following pancreas transplantation complicates accurate assessment of individual cells. An experienced immunocytologist is required to analyse the cellular debris and has to be available seven days a week for this to be a beneficial practice. Potentially, it may become employed elsewhere, as these cells could be analysed by flow cytometry, if monoclonal antibodies could specifically identify these immunoblasts.

Other methods for determining early pancreas rejection have yet to be fully evaluated. Serum C-peptide levels have been used in retrospective studies and may prove valuable as better and quicker assays become available. Pancreatic graft biopsy has been avoided in all but the most experienced centre because of the risk of complications such as haemorrhage, fistulae and pancreatitis (Sutherland *et al,* 1984). However, with the availability of fine bore needles with fine needle aspirates, this technique is gaining renewed popularity in Minneapolis.

TABLE 3.1 *COMPLICATIONS OF IMMUNOSUPPRESSION*

Infections:	Bacterial	e.g.	*Staphylococcus*
			Streptococcus
			Pneumococcus
			Haemophylus
			Pseudomonas
			Enterococcus
			Listeria Monocytogenes
			Nocardia
			Clostridium Difficile
	Specific	e.g.	*Mycobacter*
	Viral		e.g.*Cytomegalolus*
			Herpes Simplex
			Varicella Zoster
			Epstein-Barr
			Hepatitis B
			Hepatitis Non-A, Non-B
			Human Polyoma
			Papilloma
	Fungal	e.g.	*Aspergillus spp.*
			Candida spp.
			Coccidioides
			Cryptococcus
	Protozoal	e.g.	*Pneumocystis Carinii*
			Giardia Lamblia
	Helminth	e.g.	*Strongyloides*
			Schistosoma
	Other	e.g.	*Legionella*
Malignant:	Reticuloendothelial	e.g.	*Hodgkin's and Non-Hodgkin's Lymphoma*
			Plasma Cell Lymphoma
			Lymphoreticular Sarcoma
			Reticulum Cell Lymphoma
	Skin	*e.g.*	*Squamous Cell*
			Kerato-acanthoma
			Basal Cell
			Bowen's Disease
			Malignant Melanoma
	Other	*e.g.*	*Kaposi's Sarcom*
			Prostate
			Colon
			Rectum
			Breast
			Lung
			Uterus

Table 3.1 A list of some of the infections and malignant complications of non-specific immunosuppression.

3.2.3 Complications of Immunosuppression

As the incidence of technical failures following pancreatic transplantation are reduced, difficulties with diagnosis and treatment of rejection will become the major stumbling block towards success. At least then, in the foreseeable future, given that rejection episodes will still haunt transplant surgeons, alternative ways to halt this rejection process should be sought. A more aggressive immunosuppressive regimen with more aggressive anti-rejection therapy during rejection episodes could be employed, but the levels of the agents currently used are already at their safety margin threshold. Complications of immunosuppressive therapy alone contribute significantly to patient mortality and morbidity. From studies in leucaemia, neutropenia is a neglected major risk factor for bacteraemia and disseminated fungal infection, whether associated with overdosage of azothiaprine, or with cytomegalovirus infection (Bodey *et al,* 1966; Gaya, 1975). The commonly employed immunosuppressive agents include prednisone, azothiaprine, cyclosporine with anti-lymphocyte or anti-thymocyte globulin used occasionally. Indeed, the use of triple and even quadruple therapy is advocated by some, in an attempt to reduce the individual dosages of the agents while maintaining adequate immunosuppression (Sollinger *et al,* 1987). Nevertheless, morbidity and death from infection and malignancy remains a major problem in organ transplantation.

The mortality in the first year after renal transplantation is now between 5% and 10% (Krakauer *et al,* 1983) and about half of these deaths are due to infection. In the prospective series of Peterson *et al,* 1982, 32% of patients suffered a clinically significant infection; 7% of patients died and in 87% of these deaths infection was an important contributing factor. Morris reported that 58% of patients suffered an infection in the first six months after transplantation (Morris *et al,* 1982). Furthermore, the incidence of *de novo* cancer in transplant recipients can vary greatly but, nevertheless, there is a general increase in the incidence in transplant recipients. In Europe (Jacobs *et al,* 1976), *de novo* cancer development occurred in 1.6% of transplant recipients compared to 3.3% in Scandinavia (Birkland, 1983), 5.6% in an American series (Penn, 1970) and 24% in an Australian report (Sheil *et al,* 1981). If cancer of the skin is excluded there still remains an incidence of 4-7% in transplant recipients. Some of the

more common complications of immunosuppressive therapy are listed in Table 1.1. The incidence of complications due to immunosuppression is alarming and alternative methods to reduce the dosages and side effects would be most welcome.

Apart from treating the recipient and using non-specific immunosuppression, one alternative would be to render the transplanted organ itself more "acceptable" in the host and less immunogenic. The process of rejection could then be challenged from two aspects. The donor and/or the donor organ could be *pretreated*, in an attempt to reduce graft immunogenicity, i.e. reduce the stimulatory capacity of the donor tissue, and the immune response itself, elicited by the foreign tissue in the recipient, could be suppressed by conventional immunosuppression.

There have been many attempts to reduce graft immunogenicity employing a variety of novel and unique methods. However, despite achieving a significant reduction in the stimulatory capacity of the tranplanted tissue, most of these methods are not applicable to the clinical field.

3.3 IMMUNOALTERATION

3.3.1 Introduction

Reducing the antigenicity and immunogenicity of a whole organ allograft could conceivably reduce the severity and frequency of allograft rejection. Graft "immunoalteration" is a highly attractive concept if it can be achieved clinically, as it offers the opportunity of impacting on transplant survival without the risk to the recipient of conventional immunosuppression. That such immunoalteration can be accomplished experimentally has been shown in many laboratories. In 1957, George Snell first suggested that transplantable tissues contain certain haemopoeitic cells which might be important in initiating graft rejection, and that allograft survival may be greatly enhanced by the depletion of these "passenger" haemopoietic cells before transplantation (Snell, 1957). By 1971 it was clear that passenger leucocytes were responsible for the graft versus host reaction

(Billingham, 1971). Manipulation of an organ or tissue to reduce its immunogenicity, perhaps by reducing the passenger cell content within the tissue, has been attempted by several techniques. Whether or not the advantages of these pretreatment methods are due primarily to a depletion or reduction of the passenger leucocytes or, in fact, due to modulation of the antigenic expression within the graft will be discussed. Furthermore, their individual applications to the clinical setting would be of paramount importance should a significant reduction in graft immunogenicity be accomplished.

3.3.2 Pretreatment by Culture

The foundations of modern culturing techniques were greatly investigated and improved by Sir Peter Medawar. Interestingly, Alexis Carrel contributed significantly in the field of tissue culture, to which he devoted the latter half of his life. He had now given up much of his work involving whole organ transplantation presumably because of the dissappointing results of organ allografting due to rejection.

The first attempts at organ or tissue manipulation were first reported by Stone 1936 (Stone *et al,* 1934). Subsequently, Gaillard, in 1947, cultured human parathyroids for a few days prior to transplantation into patients who, following thyroidectomy, developed hypocalcaemic tetany (Gaillard, 1948). He reported six cases of "complete cure", stating that these patients became normocalcaemic due to the functioning parathyroid allografts. Even though it seemed that a period of culture benefitted the survival of the transplanted tissue, the actual immunological significance of this work was not fully appreciated until decades later.

It was not until 1967 that Jacobs and Huseby conclusively demonstrated a significant prolongation of survival of mouse tumour allografts following a period of *in vitro* culture prior to transplantation into non-immunosuppressed hosts (Jacobs and Huseby, 1967). Several investigators had previously reported that organ culture of normal endocrine tissues improved their survival in allogeneic and xengeneic hosts. However, in these reports, unequivocal evidence for the survival and recovery of the grafts was lacking (Gaillard PJ, 1954) or the grafts were placed in an immunologically privileged site (Martinovitch, 1950; 1956).

Rabotti *et al* reported that a BDF_1 ascites tumour lost its strain specificity after 30 passages in cell culture (Rabotti *et al*, 1963). The loss of histocompatibility seemed to be permanent. Decreased strain specificity of a mouse carcinoma following two serial passages on chick chorio-allantoic membrane was reported by Mirand and Hoffman (Mirand and Hoffman, 1955). In 1974, with renewed interest, stimulated by the earlier reports of Jacobs, similar work was reported by Lueker and Sharpton (Leuker and Sharpton, 1974).

The advantages of the immunological manipulation of tissue by culture were clearly seen. Jacobs extended her findings into the field of organ transplantation and published the results of the ovarian transplant experiments referred to in her initial paper. The effects of tissue culture by improved allograft survival was thus demonstrated in this murine ovarian allograft model (Jacobs, 1974). Shortly afterwards, Lafferty and his colleagues gave credence to this pretreatment phenomenon, and it is to Lafferty that the major credit for firmly establishing the value of organ culture belongs (Lafferty, 1975). He was able to demonstrate that thyroid allografts, cultured for 21 days or more with a high partial pressure of oxygen, could subsequently be transplanted to allogeneic hosts with greatly prolonged survival (Lafferty *et al,* 1975, 1976). Other workers too confirmed this phenomenon. Naji reported a reduction in the immunogenicity of isolated parathyroids (Naji *et al,* 1979), and Eloy and associates demonstrated a reduction in pancreatic islet immunogenicity following tissue culture (Eloy *et al,* 1979). Lacy not only reported similar findings with murine islet allografts, but also extended these findings in a xenograft model (Lacy *et al,* 1979 a,b).

Sollinger confirmed Lafferty's reports (Sollinger *et al,* 1977). He demonstrated that although a culture period of 10 days would prolong thyroid allograft survival, a greatly significant prolongation could be produced following 15 days of *in vitro* culture. In contrast to this, Raff and others found no effect of culturing parathyroid tissue, although they did not culture for longer than 14 days (Raff *et al,* 1974). Furthermore, the culturing conditions were different from those employed by Lafferty and Sollinger. These contrasting data suggest that many factors, including animal strain combinations, culture conditions can influence the results. Talmage and Dart demonstrated the crucial role played by a high pO_2, and were able to shorten the period of culture to 4 days when the pO_2 was increased from 600 to 1300 mm Hg (Talmage and Dart, 1978). The mechanism of the oxygen effect is not known, but Fitzsimons hypothesised that

dendritic cells are unusually susceptible to killing by oxygen free radicals and the reduction in their numbers was crucial (Fitzsimons, 1979). The concept that the reduction of immunogenicity was due to the elimination of dendritic cells as opposed to cellular or antigenic-protein changes within the tissue was addressed to an extent by Talmage. He argued that injection of leucocytes syngeneic with the graft would cause accelerated rejection only if depletion of tissue leucocytes was responsible. In 1976, he confirmed his hypothesis and elicited acute rejection of accepted thyroid allografts with an injection of donor leucocytes (Talmage *et al,* 1976). Lacy produced a similar effect on islet allograft with and injection of donor peritoneal Class II MHC antigen-bearing macrophages (Lacy *et al,* 1979b), and this effect was further proven by similar experiments in long standing kidney allografts (Lechler and Batchelor, 1982)

The inherent advantages of this "culturing" phenomenon, however, were overshadowed for a while with the discreditation of Summerlin, who had previously reported that a period of culture made it possible to graft skin to allogeneic mouse or human recipients (Hixson, 1976). The incident made other workers re-evaluate their findings. Nevertheless, despite this set back, research continues to elaborate on the immunological advantages of tissue culture.

The beneficial effects of organ culture before transplantation has been attributed to depletion of viable haematogenous elements and lymphoid cells, and/or a decrease in the concentration or availability of transplantation antigens (Ia) on the cell surface membranes. There is histological evidence that that passenger cells and vascular endothelium disappear from mouse islets after 7 days of culture (Parr *et al,* 1980). Rabinovitch and colleagues have shown that lymphocytes, macrophages, and also capillary endothelium are reduced by culture, and that this is accompanied by a significant decrease of Ia antigen density as determined in a sensitive radioimmuno-binding assay (Rabinovitch *et al,* 1982).

The temperature of the culture environment has been studied in some detail in Lacy's laboratory. They found that the survival of rat islet allografts, transplanted across a major histocompatibility barrier, could be prolonged to greater than 100 days if the islets were maintained in culture at 24°C, rather than 37°C, for seven days prior to grafting (Lacy *et al,* 1979c). It is interesting that Opelz and Terasaki reported that lymphocytes lose their ability to stimulate

allogeneic cells following a 4 day culture at 22°C (Opelz and Terasaki, 1979). Lacy and co-workers extended their findings to a rat to mouse xenograft model, and together with Ricordi, reported similar results with intra-testicular islet allografts and adrenal cortical allografts (Ricordi *et al*, In Press).

Finally, the use of tissue culture has been applied to fetal tissue transplantation. The possibilities of curing experimental diabetes mellitus in animals by using syngeneic transplantation of fetal pancreases has been well documented (Mullen *et al*, 1976; Sutherland *et al*, 1976; Gray *et al*, 1986). Successful experiments have prompted investigations to explore the usefulness of human fetal pancreas for transplantation to diabetic patients (Sandler *et al*, 1985). Collier and Mandel reported a reduction of mouse fetal pancreas immunogenicity by culturing in 90% oxygen (Collier and Mandel, 1983), but later added that a combination of both low temperature and high pO_2 may infact be damaging to the fetal islets as well as the dendritic cells (Mandel and Koulmanda, 1985).

Whatever the effects and advantages of tissue culturing may be, the techniques employed to individual cells or endocrine tissue grafts cannot be directly applied to whole organs. Whether the effect is due to antigenic modulation within the allograft or due primarily to the elimination of the passenger leucocytes is still debated. If temperature and oxygen tension are indeed important factors, better and alternative techniques of preservation need to be developed for application to whole organs. Nevertheless, the fact that a variety of culturing methods, applied to transplantable tissues, results in significant allograft prolongation, gives great strength to the arguement that *pretreatment* can infact reduce graft immunogenicity. With this in mind, a pretreatment regimen which could reduce the immunogenicity of whole organs, would be obviously beneficial.

3.3.3 Pretreatment by Irradiation

The radiosensitivity of lymphoid cells was well established with the improvement in survival of patients with Hodgkins lymphoma treated with irradiation. In 1902, Pusey was able to demonstrate immediate improvement using local irradiation in these patients (Pusey, 1902).

The lymphocytic and plasma cell infiltration into the interstitium of the allograft is one of the earliest and most characteristic histological changes of rejection. Irradiation, either as pretreatment or adjuvant immunosuppression seemed therefore to be a logical step. The application of *whole body* irradiation to transplant immunology was first attempted in the late 1950's when prospective renal allograft recipients were pretreated. Therapy aimed at prolonging functional survival of renal allografts consisted primarily of total body irradiation (TBI) and systemically administered immunosuppressive agents. Murray used total body irradiation with or without bone marrow in 12 patients (Murray *et al,* 1962), while Hamburger employed total body irradiation (TBI) alone in his first 4 patients and did not use steroids until the one hundredth day post transplant in his fifth patient (Hamburger *et al,* 1962). TBI alone or in combination with irradiation of the spleen was utilised by Kuss in 3 of his first 6 patients. Hume, Kuss and Shackman used varying combinations of TBI and drugs in their renal transplant recipients (Hume *et al,* 1963; Kuss and Legrain, 1962; Shackman *et al,* 1963). Hume and his associates then introduced local graft irradiation in combination with drugs (Hume *et al,* 1963), and Woodruff used drugs and graft site irradiation (Woodruff *et al,* 1963). Sublethal doses of TBI commonly employed not only suppressed production of haemopoeitic cells but also impaired the primary immune response to a variety of antigenic stimuli temporarily. However, it was found that the irradiation dose required to produce allograft prolongation also produced such severe bone-marrow and gastro-intestinal damage that with the introduction of pharmaceutical immunosuppressions, whole body irradiation was abandoned (Hamburger *et al,* 1962).

It wasn't, however, until it was shown by Fuks and his colleagues that there was a substantial deficiency in the T cell function in patients suffering from Hodgkins lymphoma, following local lymphoid irradiation (Fuks *et al,* 1976), that led Slavin and Strober to consider the use of total *lymphoid* irradiation as an immunosuppressive agent in transplantation (Slavin *et al,* 1979), rather than TBI. Kauffman, in 1966, demonstrated that local graft irradiation in dogs, following transplantation, significantly prolonged renal allograft survival (Kauffman *et al,* 1966) but a synergistic effect better than that with conventional immunosuppression alone has yet to be reported clinically.

The evidence that irradiation may be advantageous as *pretreatment* is limited. The usefulness of pretreating donors or donor tissue was investigated by

several authors. Stuart confirmed that irradiation of the intact donor (rat and dog) prolonged allograft kidney survival without additional immunosuppression to the recipient (Stuart *et al,* 1971), but that it was only effective if the irradiation *preceded* transplantation by an interval long enough to allow the appearance of profound peripheral leucopenia. This was usually 48 to 96 hours. McKenzie and colleagues also demonstrated prolonged survival of cardiac allografts following pretreatment with irradiation to the donor animal (McKenzie *et al,* 1984). Similarly, Freeman verified that whole body irradiation 3-5 days before transplantation, resulted in significant cardiac allograft survival (Freeman *et al,* 1971). Local irradiation to the heart, however, did not have any effect on transplant survival. In the corroborating studies, which demonstrated a benefit of allograft prolongation, to achieve any significant effect, the irradiation had to be given at least 3 days prior to transplantation. Hume *et al* could show no benefit on canine renal allograft survival when the donors were treated with 1000 - 1500 rads 1 day before donor nephrectomy (Hume *et al,* 1963). The realisation that the benefits of allograft or donor pretreatment with irradiation occur only after three days, is disappointing because this regimen is therefore not applicable to the clinical situation and whole organ transplantation.

3.3.4 Pretreatment with Drugs

Guttmann and his colleagues reported that pretreating donor rats with anti-thymocyte globulin (ATG), kidney allograft survival was prolonged (Guttman *et al,* 1967). Furthermore, he also demonstrated a beneficial effect of pretreating donors with high dose cyclophosphamide (Guttmann and Lindquist, 1969). His results were confirmed by Steinmuller's group who showed that donor pretreatment with cyclophosphamide, 100 - 200 mg/kg intramuscularly, one to four days prior to transplantation, resulted in significant allograft survival (Freeman *et al,* 1971). He reported similar results using antilymphocyte serum. McKenzie, Beard and Hart pretreated rats with either cyclophosphamide or irradiation, or both. They confirmed that all the pretreatment regimens were effective in prolonging rat cardiac allograft survival (McKenzie *et al,* 1984). Their explanation for the effect was the decrease in the numbers of Ia +ve cells

within the heart, but should more than 5% remain following pretreatment, there would not be significant survival. Nielson *et al*, in contrast, reported that removing these accessory cells from rat hearts had no effect on allograft survival (Nielsen *et al*, 1975). However, issue could be taken with their methodology. First, in their ALG pretreated group, they simply flushed the hearts at 4°C prior to transplantation. There are no reports that this simple flushing technique reduces or eliminates the numbers of interstitial dendritic cells. Furthermore, in their alternative method, using an enhanced intermediate host, the time of residence was only 72 hours. Several authors have shown that 3 weeks or more are necessary to achieve replacement of these specialised cells with those of host origin (Stuart *et al*, 1976; Lechler and Batchelor, 1982).

It is not only cytolytic drugs which have been employed to pretreat organs. Recently, Toleda-Pereyra reported prolonged survival of canine renal allografts after pretransplant flushing of the donor kidney with 250 mls of lactated Ringer's solution containing 50 mg of cyclosporine (CyA)(Toleda-Pereyra *et al*, 1982). This work has also been corroborated by some (Rucker *et al*, 1982), but not by others (Jeng *et al*, 1986). Gruner and his co-workers reported a significant prolongation of murine skin allografts after donor pretreatment with CyA 24 hours before graft procurement (Gruner *et al*, 1986). It was suggested that the action of CyA pretreatment was dependent on the prevention of the induction of MHC Class II antigen expression and a reduction in graft immunogenicity.

If the pretreatment of whole organs with many of the agents described above reduces the immunogenicity of the allograft, their effects, in part, are probably mediated through the depletion or inactivation of the Class II bearing dendritic cells within them. However, to ascertain whether the depletion of these cells *alone* is the only parameter necessary to achieve allograft prolongation, a more specific effect can be examined by the pretreatment of organs with specific anti-Class II monoclonal antibodies.

3.3.5 Pretreatment with Anti-Ia Monoclonal Antibodies

It had been previously demonstrated that *in vitro* stimulation of cell mediated immunity depended on Ia bearing macrophages or dendritic cells

(Yamashita and Shevach, 1977; Silberberg-Sinakin *et al,* 1980). In 1976, Davies and Staines demonstrated enhancement of skin allografts following pretreatment with Ia antisera (Davies and Staines, 1976). Shortly afterwards, McKenzie and Henning demonstrated probable enhancement of renal allografts by similar pretreatment (McKenzie and Hemming, 1977).

Lacy's group demonstrated that pancreatic islet β-cells lacked Class II MHC antigenic determinants on their cell surface with the premise that pretreatment with anti-Ia sera would not damage the endocrine-functioning cells within the pancreatic islets; only the interstitial Class II bearing macrophages and dendritic cells would be eliminated (Faustman *et al,* 1980). In 1981, Denise Faustman, together with Lacy, published data confirming that pretreatment of murine islet allografts with anti-Ia sera resulted in significant allograft survival (Faustman *et al,* 1981). Their conclusions were that the pretreatment regimen employed removed the contaminating dendritic cells which eliminated the immunogenic component of the islets. Reece-Smith, at Oxford, similarly attempted pretreatment of rat pancreatic islets (LEW to DA) using a monoclonal antibody reactive with Class II MHC antigens (Reece Smith *et al,* 1983). However, although a prolongation was reported, it was not significant. First, this may have been because the monoclonal antibody used was F17.23.2 which has been reported not to fix complement effectively (Hart and Fabre, 1981); second, in the experiments, the islets were incubated at room temperature (20 - 24°C), so complement-dependent lysis of the Class II antigen bearing cells would have been minimal, should binding of the antibody have occurred.

Otsubo and Yamamoto *et al* were the first to attempt whole organ pretreatment with anti-Class II monoclonal antibodies (Ostubo *et al,* 1983). They flushed isolated dog kidneys with 10 mls of HAK-75, a γ 2b monoclonal antibody reactive against splenic B cells of H-2^k mice, and incubated the organs at 4°C for 30 mins following clamping of the artery and vein. Significant allograft survival was achieved following this procedure although criticism could be raised because they used outbred dogs. Nevertheless, despite this and despite incubating at 4°C, at which temperature only labelling of the antibody, and no C'-dependent lysis could occur, a reduction in graft immunogenicity, as demonstrated by a prolongation of allograft survival, was achieved. Pollak, on the other hand, could not show any benefit upon allograft survival by simple

flushing of mouse hearts or rat kidneys with anti-Ia antisera or a specific anti-dendritic MAb, 33D1 (Pollak *et al,* 1987). Again, these experiments were performed at 4°C.

Asano and is co-workers, attempting to increase the time of exposure of the Class II positive cells to the antibody, studied the effects of prolonged pulsatile perfusion of canine segmental pancreases and added an anti-Ia monoclonal antibody, ISCR3, to the perfusate (Asano *et al,* 1985). In these experiments, they were unable to demonstrate significant allograft prolongation, but commented that there were difficulties in assessing the correct dose of MAb to use and postulated that the permeability and cytolytic activity of this MAb should be investigated further before negative conclusions be drawn.

To investigate the effect of dendritic cell depletion from intact islets, Steinman developed a specific monoclonal antibody directed against mouse dendritic cells and collaborated with Lacy's group to pretreat murine islets (Steinman *et al,* 1983; Nussenzweig *et al,* 1980). In 1984, Faustman *et al* demonstrated that the rejection of fully allogeneic mouse islets could be abrogated when pretreated with the anti-dendritic cell monoclonal antibody, 33D1, and rabbit complement (Faustman *et al,* 1984). There was increasing evidence that elimination of Class II positive passenger leucocytes reduced graft immunogenicity.

However, in 1985, Morrow and Sutherland retracted comments previously published in 1983 (Morrow *et al,* 1983), that pretreatment of pancreatic islets with anti-Ia antisera or MAb had any significant effect on allograft survival (Morrow *et al,* 1985). Furthermore, in contrast to Faustman's studies, they reported that there was no effect on allograft survival following pretreatment with anti Ia anti-sera (Gores *et al,* 1985). However, the strain combinations employed were different and the islets were placed beneath the renal capsule in contrast to being injected into the portal vein in Faustman's experiments. The presentation of the alloantigens may differ in both these sites which could be fundamentally important in determining the host response and may explain the absence of an effect in the renal subcapsular site.

The differences in the effects of anti-Ia pretreatment may indicate the difficulties in eradicating the Class II positive cells from the donor tissue. The effect, if any, may also be masked by MHC strain differences, but the most

important determination is to establish whether complete removal of the passenger cells was accomplished before transplantation. McKenzie strongly suggests that if only 5% of dendritic cells remain, then there will be sufficient Class II antigen presentation to initiate rejection and consequently no immunological effect will be apparent (McKenzie *et al*, 1984). Stringent analytical methods should be employed to determine the effectiveness of the attempts at Ia positive cell depletion. The studies in which no prolongation of allograft survival was reported, and where no confirmation of the complete removal of the Class II positive leucocytes prior to transplantation was established, have been criticised by Lafferty (personal communication).

If the effect of removing passenger cells by anti-Ia pretreatment alone, is not sufficient to abrogate rejection, then a synergistic effect with adjuvant immunosuppression might be demonstrated. Terasaka reported a beneficial and synergistic effect on allograft survival of rat islets, following a combination of pretreatment with anti-Ia antibodies and a short 3 day course of recipient immunosuppression with cyclosporine (Terasaka *et al*, 1985). More recently, Alejandro *et al* confirmed that even though there was a beneficial effect of pretreating canine islets with anti-Class II MAb, a synergistic improvement on allograft survival was achieved following a combination with low dose cyclosporine (Alejandro *et al*, 1986).

These reports suggest that there is a reduction in graft immunogenicity following pretreatment with anti-Ia MAb, but the results are variable. The elimination of the Class II positive cells from the tissues must be optimised if any benefit upon allograft survival is to be achieved. For the application of anti-Ia MAb pretreatment to whole, vascularised organ allografts, therefore, the dendritic cells within the organs must be exposed to the anti-Class II agents for a prolonged period of time, and in order to utilise the effects of complement lysis, the exposure must be at 37°C. In this dissertation, the development of an oxygenated, normothermic perfusion circuit to deliver monoclonal antibodies to the intact rat pancreas will be described and the effects of prolonged perfusion with anti-Class II MAb and C' reported.

3.3.6 Pretreatment using Ultraviolet Irradiation

It appears from many of the above studies that the abolition of the original recognition of an allograft may be achieved by the elimination of Ia positive cells from within the allograft and that it is critical to successful allografting without further immunosuppression. It further appears that immunisation with Ia-negative donor blood cells induces immunologic unresponsiveness to the donor strain in the recipients by mechanisms that have not yet been defined, but that may be caused by the stimulation of specific suppressor cells (Hardy *et al,* 1984). Since it was known that ultraviolet (UV) irradiation of the stimulating cell population in a primary mixed lymphocyte reaction lead to minimal or absent proliferative response (Hayry *et al,* 1976), Hardy postulated that the Ia positive cell may not necessarily need to be eliminated from either the islets or from blood prior to its use for immunisation, but may need to be inactivated with UV light (Hardy *et al,* 1986).

In a series of experiments, it was shown that pretreatment with UV irradiated donor blood resulted in significant allograft prolongation of pancreatic islets (Hardy *et al,* 1984). Furthermore, actual pretreatment of islets themselves with UV irradiation, produced allograft prolongation upon transplantation (Hardy *et al,* 1984). This phenomenon was thought to be due to the elimination or inactivation of the interstitial Ia positive dendritic cells. However, the results were extremely dependent on the strain combination of animals used. ACI ($RT1^a$) rats were used as diabetic recipients and Lewis (LEW, $RT1^l$) or Wistar Firth (WF, $RT1^u$) animals used as donors. UV irradiation used in other strain combinations has not been successful in achieving similar results.

Recently, however, the application of UV irradiation to whole organs has been reported to produce a significant improvement in allograft survival (Oesterwitz *et al,* 1986). These workers pretreated rat renal allografts with 8-methylpsoralen and subsequently irradiated the grafts with UV light, thus reducing graft immunogenicity by photochemical inhibition of the passenger leucocytes. This work is yet to be confirmed by others.

3.3.7. Immunoalteration during preservation

Attempts at modifying organ allografts by pharmacological manipulation during storage derive from the work of Guttmann in the early 1970's. Pretreating the donor with lympholytic agents such as cyclophosphamide, steroids and anti-lymphocyte serum was shown to prolong graft survival. Recent work at the Mayo clinic (Zinke *et al,* 1978), with experimental evidence in animals (Oluwole *et al,* 1980; Van der Linden *et al,* 1980; McCabe *et al,* 1980) confirm the effectiveness of Guttmann's extensive clinical work. Initial experiments by Woods, however, failed to demonstrate any prolongation of canine renal allografts when azothiaprine, methyl prednisolone and phytohaemagglutinin (PHA) were added to the cryoprecipitated plasma in a Belzer renal preservation system (Woods *et al,* 1977). Guttmann, however, then showed that when kidneys were pretreated *in vivo* in the donor animal their subsequent survival as allografts was greatly improved if they were also perfused with plasma prepared from animals which had themselves been pretreated. In addition to lympholytic drugs, the passenger cell hypothesis suggested that perfusion with anti-lymphocytic serum (ALS) may be useful. Callender and co-workers perfused canine kidneys at 7°C with antilymphocyte globulin for one hour and reported significant allograft survival if azothiaprine was also given to the recipients (Callender *et al,* 1973).

Other *in vitro* treatments have also been studied. The knowledge that the plant lectins Concavalin A (Con-A) and PHA mask histocompatability antigens on mouse lymphoid cells, led Toleda-Pereyra to perfuse canine kidneys with solutions containing these substances prior to allografting (Toleda-Pereyra *et al,* 1974). He showed that there was a significant prolongation in allograft survival following this pretreatment.

Many of the other attempts at immunoalteration have been applied to isolated cells or tissue. These methods cannot be directly applied to intact whole organs. Indeed, the use of cytotoxic drug pretreatment, such as cyclophosphamide or methotrexate, or irradiation to whole vascularised organ grafts needs to be applied several days prior to organ retrieval (Stuart *et al,* 1971). To have any impact in the clinical field, a system should be developed which could reduce graft immunogenicity within hours of the pretreatment regimen. The

attempts at significantly reducing graft immunogenicity by pretreating whole organs with immunological agents, such as ALG or ATG, have involved simple flushing of the organs just prior to transplantation, at 4°C. If elimination or inactivation of the Class II antigen bearing cells within the graft is paramount for the reduction of immunogenicity, then the application of *complement dependent 'inactivation'* has an obligatory temperature dependence. The tissue must be perfused at normothermic temperatures (37°C) for optimal lysis of the Class II positive cells by complement fixation.

3.3.8 Immunoalteration of Pancreatic Islets Versus Whole Pancreas Allografts

Immunoalteration of experimental pancreatic islet allografts most clearly suggest a central role for donor dendritic cells in allogeneic immunisation. As previously discussed, culturing isolated rodent islet cells at high oxygen concentrations (Lacy *et al,* 1979), exposing isolated islets to ultraviolet irradiation (Hardy *et al,* 1984), or treating isolated islets with anti-sera specific for class II MHC antigens (Faustman *et al,* 1981), all treatments known to be cytotoxic for dendritic cells, has each resulted in marked and often indefinite prolongation of islet cell allografts.

Unfortunately, while transplantation of pancreatic islets can reverse experimentally induced diabetes in the rodent, islet transplantation in the human has been plagued with difficulties in retrieving adequate numbers of viable islets to produce insulin independence after transplantation. In contrast, with recent refinements in techniques such as the use of a duodenocystostomy and with the use of multiple drug immunosuppressive drug regimens, the survival of human pancreas organ allografts has improved greatly. Whereas only 16 percent of pancreas organ allografts performed before 1983 produced insulin independence in recipients for as long as one-year, the success rates have increased steadily since then such that many centres are reporting one year pancreatic graft survival rates of over 70% (Groth, 1988).

However, at present the most frequent cause of pancreas organ graft loss remains graft rejection, accounting for greater than 50% of failures. Thus a viable

method for immunoalteration of the intact pancreas which could reduce graft antigenicity and/or immunogenicity, and decrease the the frequency of rejection could lead to even further improvement in pancreas transplant results. The use of anti-Class II MAb's to pretreat whole human pancreases, as opposed to individual rodent islets, is limited because of the inherent differences in antigen expression on the different tissues. Faustman reported that on all β cells from the mouse the H-2K and H-2D antigens, but not the Ia antigens, are expressed on the islets of Langerhans (Faustman *et al,* 1982). Immunohistochemical analysis performed in order to localise Class II MHC antigens on β cells from human pancreases showed that the major site of DR expression in the islet was on the endothelial cell surface (Alejandro *et al,* 1982). All other tissues, including endocrine, acinar and ductal cells, were entirely devoid of DR expression. Because the islet is intimately associated with an extensive meshwork of capillaries, the use of anti-Class II MAb's, in an attempt to reduce the graft immunogenicity of human pancreases, would probably result in islet destruction, and vascular complications. However, the Class II positive dendritic cells in the pancreatic interstitium, also express the leucocyte common antigen (Steinman and Nussenzweig, 1980). The elimination of the passenger leucocytes could therefore be accomplished with a complement fixing MAb reactive with this antigen.

In addition, if the methods employed in reducing the immunogenicity of pancreas organ allografts were successful, such a method of immunoalteration could be adapted for use with other solid organs.

3.4 DENDRITIC CELLS

There has been great interest in what cells and what cellular antigens actually stimulate an immune rejection response. While Class I antigens of the major histocompatability complex (MHC) are expressed on most cell types, Class II antigens are expressed constitutively on certain leucocytes and vascular endothelium in man. In the rodent, vascular endothelium appears to lack Class II antigens when evaluated by several laboratories (Hart and Fabre, 1981; Lloyd *et*

al, 1987; Parr *et al,* 1982), although a low level of Class II antigen expression has been detected on capillary endothelium in some strains by one group (Ulrichs *et al,* 1987), and on mouse pancreatic endothelium in by others (Natali *et al,* 1981).

Donor and host incompatibilities for Class II MHC antigens clearly appear necessary for the development of a maximal allogeneic primary immune response. Thistlethwaite and colleagues have shown that kidney grafts in the pig can survive indefinately without immunosuppression despite Class I MHC incompatabilities if animals are matched for Class II antigens (Thistlethwaite *et al,* 1983). Also, others have shown that pancreatic islet grafts in the mouse can survive indefinitely without immunosuppression despite Class I MHC incompatibilities, if animals are matched for Class II antigens (Morrow *et al.,* 1983). In the rat, matching for donor and recipient Class II antigens also results in marked prolongation of survival of pancreas organ allografts, although, in the strain combinations employed, these grafts were eventually rejected (Klempnauer *et al,* 1983). The probable reason the Class II MHC antigens are necessary to elicit a maximal rejection reaction is that, in most experimental transplant models, these antigens lead to stimulation of the T helper cell which appears to be the pivotal cell type in generating an immune response. Ideally, if all cells which expressed Class II MHC antigens could be eliminated from a graft, its immunogenicity would be greatly reduced. However, it may not be necessary to remove all Class II antigen bearing cells from a graft to reduce its immunogenicity. In a mixed leucocyte culture, which may be an *in vitro* correlate to the early phase of acute rejection, leucocytes of a donor are used to stimulate those from a responder, and cell proliferation taken as an indication of the strength of the allogeneic stimulation. Steinman has shown that depleting a donor spleen cell population of dendritic cells, but not macrophages or B cells which also express Class II antigens, inhibits responder cell proliferation in murine mixed leucocyte cultures (Steinman *et al,* 1983).i.e. Dendritic cells are potent stimulators of the primary immune response.

Dendritic cells (DC's) are a morphologically distinct population of bone marrow derived leucocytes that are found both in the circulation and in the interstitia of most organs (Hart and Fabre, 1981). The frequency of DC's in all tissues is small, less than 1%, but the cells have been detected in lymphoid and most non-lymphoid tissues apart from brain (Hart and Fabre, 1981). As

reviewed by Steinman, they are sensitive to oxygen toxicity, to ultra-violet and ionizing radiation, and to steroids and cytotoxic drugs (Steinman and Nussenzweig, 1980). Their labelling index is only 1.5-2.5% 1 hour after an intravenous or intraperitoneal pulse of [^{3}H]thymidine, indicating that they are not rapidly proliferating. Similar results were obtained when splenic dendritic cells were exposed to [^{3}H]thymidine *in vitro*, differentiating them from large lymphocytes which label with a much higher frequency (Steinman *et al,* 1974). Despite their low rate of proliferation, however, DC's appear to undergo substantial turnover, i.e. following pulsing or labelling with [^{3}H]thymidine, labelled DC's rapidly replace non-labelled ones. Following a pulse of [^{3}H]thymidine (a single intra-peritoneal injection or four intra-muscular injections over 12 hours), labelled DC's appeared at a constant rate of 4-6% per day for 5 days, and then began to disappear (Steinman *et al,* 1974).

Using monoclonal antibodies (MAb) as reagents to label surface antigens, DC's are found to express larger amounts of Class II MHC antigens per cell than any other leucocytes. They lack surface immunoglobulin and Fc receptors; however, they do express Class I MHC antigens and the leucocyte common antigen. Dendritic cells are irregularly shaped cells and do not exhibit any phagocytic or endocytic capabilities which distinguishes them from classical macrophages. Furthermore, DCs have only partial adherent properties in contrast to macrophages. It is this property which enables their relatively simple isolation. Classical macrophages adhere to glass and plastic quite firmly and for prolonged periods. DCs, on the other hand, loose their adherent quality in an 18 hr culture, during which time they become freely suspended in the culture medium. Not all DCs seem to have this peculiar property. Recently, Shah, at the University of Chicago, demonstrated that DCs isolated from mouse livers were more characteristic of the macrophage. In these experiments, hepatic DCs were firmly adherent even after 48 hr of culture at 37°C (Shah *et al,* In Press).

In addition, in the mouse, dendritic cells do not appear to react uniformly with monoclonal antibodies which detect macrophage specific antigens. However, rat dendritic cells have been shown to be labelled with monoclonal antibody, W3/25, which also reacts with macrophages and T helper cells (Darden *et al,* 1987); human dendritic cells share surface antigens with macrophages as detected by at least two difeerent MAb's FMC32 and KB90 (Franklin *et al,* 1986).

The role of dendritic cells in the immune system is not totally understood, although participation of this cell type seems crucial in the initiation phase of an immune response. *In vitro*, dendritic cells appear to be a requisite accessory antigen presenting cell in the development of a maximal allogeneic proliferative and cytotoxic responses (Steinman *et al,* 1983). *In vivo*, the dendritic cell normally appears to process and present foreign antigens to the helper T cell. However, in organ allografting, the donor dendritic cell may be uniquely able to directly present its own alloantigens, including Class I antigens, to recipient T cells. In the absence of donor dendritic cells, both Class I and Class II antigens of the donor may require processing by the recipient's accessory cells before being effective stimulants of helper T cells. Lechler and Batchelor have argued that this second route of alloantigen presentation may be less effective and efficient route of sensitisation similar to immunisation to any foreign antigen and one perhaps more easily controlled with standard immunosuppression (Lechler and Batchelor, 1982a).

These conclusions about the central role for dendritic cells in stimulating an *in vivo* allograft rejection response are based on observations made in models involving transplantation of rat kidneys first to enhanced recipients to allow i*n vivo* passenger cell depletion and then to a secondary non-immunosuppressed recipient. Using a donor-recipient strain combination which normally results in indefinite graft survival in the secondary recipient, Lechler and Batchelor have shown that injection of this secondary recipient with as few as 10^4 donor leucocytes from a suspension enriched with dendritic cells leads to acute rejection of the graft (Lechler and Batchelor, 1982b). In contrast, cell suspensions enriched with T or B cells were two log orders of magnitude less potent in producing this result. Adherent monocytes, which are known to be a mixture of phagocytic and dendritic cells, cause rejection at an intermedite dose.

It is important to note that the strain combinations employed in these experiments are crucial to the extent of prolongation of graft survival. In the (AS x AUG) F_1 donor and AS recipient pairs reported by Batchelor, grafts are routinely accepted by the secondary host. In the (LEW x BN) F_1 donor and LEW recipient combination studied previously in Stuart's laboratory, greater than half the secondary host animals died of uraemia in less than 15 days following native nephrectomy and transplantation (Stuart *et al,* 1971); the rest continued to

have long term survival. In the DA to LEW, (LEW x DA)F_1 to LEW, and (LEW x DA)F_1 to DA donor - recipient pairs that were studied by Hart and colleagues, grafts in the first combination were promptly rejected by unsensitized secondary recipients even after passage through an enhanced primary host; grafts in the second combination underwent a less vigorous rejection with some surviving long term; and those in the third combination survived indefinitely in the secondary recipient without evidence of impairment of graft function (Hart *et al,* 1980).

McKenzie made the observation that grafts from different rat strains differ in the number of dendritic cells they contain by up to a factor of 3 (McKenzie *et al,* 1980). They maintain that dendritic cells are more completely eliminated by pretreatment regimens from organs of strains with a lower initial dendritic cell count and that a 95% depletion is necessary for prolonged survival, accounting for the survival differences in different donor-recipient strain combinations. An alternative explanation, which is equally plausible, is that alternative mechanisms of donor antigen presentation by recipient dendritic cells may play a larger role in rejection in some strains.

3.5 CONCLUSIONS

These reports and observations centre around the Class II positive dendritic cell as being extremely important in evoking a primary allogeneic immune response *in vitro* and *in vivo*. Moreover, a pretreatment regimen which could completely eradicate these passenger cells from a whole vascularised organ could greatly reduce the immunogenicity of an allograft. Whether rejection episodes will be eliminated by such removal, or that the rejection episodes will be less severe and less frequent is as yet undetermined and remains contoversial. There has been no pretreatment regimen employed so far that can *completely* eliminate all Class II positive cells from a vascularised organ allograft. Until this is accomplished the hypothesis, that their removal will result in a significant reduction in graft imunogenicity, will remain unproven.

SECTION II

Materials, Methods and Experiments

CHAPTER 4

ANIMALS AND MONOCLONAL ANTIBODIES

4.1 INTRODUCTION

The work proposed was to establish whether intact organs could be perfused in an *ex vivo* situation with anti-Class II monoclonal antibodies, and determine the effects on allograft survival. It is apparent that if any significant reduction in graft immunogenicity was to be achieved by the proposed *ex vivo* pretreatment regimen, then choosing a strain combination which had already been shown to be 'enhancable' would initially be the logical choice of animal strain combination for these experiments. Any benefit of immune alteration by the pretreatment of pancreatic allografts may be overcome by the vigor of rejection using a fairly strong rejecting strain combination such as Lewis x Brown Norway ($RT1^{l,n}$) to LEW ($RT1^{l}$) rats. Stuart *et al* were able to demonstrate immunological enhancement of *renal* allografts using cells and serum in this strain combination. They proposed that the prolongation of allograft survival was due to the induction of synthesis of anti-receptor antibody in the host by pretreatment of the host with donor-specific antigen and host anti-donor antigen (Stuart *et al,* 1976). However, when the same pretreatment protocol was used for pancreas transplantation in this strain combination, no significant immunological enhancement could be demonstrated, as crudely measured by graft survival (Reckard *et al,* 1981). An alternative combination had to be sought.

The LEW to DA barrier is known to be weaker one than the DA to LEW barrier in regard to kidney transplantation (Fabre and Morris, 1974). Graft adaption was clearly more demonstrable in this strain combination, and indeed was further studied by Hart. He and colleages reported definite allograft enhancement in the survival of kidney allografts, transplanted from the Lewis (LEW) and Dark Agouti (DA) hybrids ($LDAF_1$), into DA rats when the original organs were retransplanted from long standing immunocompromised hosts (Hart *et al,* 1980). Furthermore, in a similar strain combination, AS x DA hybrids to DA recipients, a significant prolongation of allograft survival was achieved when the donor animals were pretreated with cyclophosphamide (McKenzie *et al,*

1984). The availability of $LDAF_1$ rats and the previously published by Morris and Fabre that possible graft adaption was possible in this strain combination, were the main indications for using this particular strain combination in the transplantation experiments presented here.

4.2 ANIMALS USED

The animals used in all the experiments were male rats weighing between 180 -250 grams. The Lewis animals (LEW), with an MHC complex haplotype designated $RT1^l$, were purchased from Harlan Sprague Dawley, Indianapolis, Indiana; the Dark Agouti rats (DA), with haplotype $RT1^a$, were obtained from The Trudeau Institute, Saranac Lake, N.Y.; similarly, the Lewis and Dark Agouti hybrids ($LDAF_1$), haplotype $RT1^{a,l}$, were also purchased from the Trudeau Institute. The animals were tested for routine viral and parasitic infections before being sent and quarantined for seven days on arrival.

4.3 INDUCTION OF DIABETES

4.3.1 Introduction

There are a variety of techniques described to induce a state of diabetes in experimental animals. Complete removal of the gland dates back to 1893 (Von Mering and Minkowski, 1889) and 1922 (Banting and Best). The technique has been well described in the dog (Markowitz *et al,* 1964), but also in the rat (Scow, 1957) and monkey (Mirsky *et al,* 1937). However, for simplicity, in the rat model, where a study of the endocrine function of the gland is required, if the exocrine function of the pancreas could be preserved, then only the insulin producing pancreatic islets could be transplanted, instead of the whole organ. Moreover, if the intact rat pancreas was transplanted into a diabetic animal with its own exocrine portion of the gland functioning, then the added complications of handling the ductal drainage could be ignored. The induction of diabetes by chemical means was therefore extremely advantageous in the rat.

4.3.2 Streptozotocin

In 1943, Dunn and his associates reported that alloxan produced hyperglycaemia in rabbits and later went on to report the use of alloxan to induce diabetes in the rat (Dunn *et al,* 1943). Alloxan itself is extremely toxic, and in 1963, a new and safer agent was described to be diabetogenic in animals.

Streptozotocin, an anti-bacterial agent isolated from the fermentation cultures of *streptomyces achromogenes*, has become the standard drug for causing diabetes in rodents (Rakieten *et al,* 1963). The compound itself is the 1-nitroso-1-methylurea derivative of 2-deoxyglucose; it is the 1-nitroso-1-methylurea part of the molecule which is diabetogenic (Gunnarsson *et al*, 1974). The actual biomolecular reasons why it damages the islets is thought to be associated with the irreversible depletion of the NADH in the pancreatic β cells, since nicotinamide blocks the effect (Schein and Loftus, 1968). The response to a single injection of streptozotocin is triphasic: the initial hyperglcaemia occurs 1-2 hours after injection, followed by insulin release at 6-12 hours resulting in transient hypoglycaemia, and finally permanent hyperglycaemia occurring 24 hours after streptozotocin administration. Histological examination reveals severe β cell necrosis. Dose response studies in the rat established that the severity of diabetes produced is directly related to the dose injected. A dose of 100 mg/kg i.v. produced severe hyperglycaemia, ketosis and death within 3 days, whilst a dose of 35 mg/kg i.v. resulted in mild diabetes with spontaneous recovery observed in 25% of animals. A dose of either 55 mg/kg or 65 mg/kg i.v. resulted in moderately severe diabetic state with hyperglycaemia, polyuria, moderate ketosis and weight loss which was stable and compatible with survival for several weeks.

Following one week in isolation and quarantine, the animals selected to be recipients of either an intact pancreatic graft or pancreatic islets were injected with streptozotocin (a kind gift from Upjohn Co., Kalamazoo, Michigan, USA) 65 mg/kg intravenously in the penile vein. The drug was dissolved in normal saline. The blood sugars were monitored intermittently for three weeks prior to transplantation to confirm a high blood sugar. Levels of 300 mg/dl or above were

taken as proof of diabetes, although to avoid the occassional reversion to normal glycaemia which occasionally occured, blood sugars were always taken immediately prior to transplantation.

4.4 ANIMAL SURGERY - PANCREAS TRANSPLANTATION

4.4.1 Donor Pancreatectomy

The animals were anaesthetized with an intra-peritoneal injection of chloral hydrate (30 mg/ml) and the abdomen shaved and cleansed with either 70% alcohol or povidone iodine. Following a midline incision the intestines were wrapped in gauze soaked in Neomycin-Saline, and careful dissection of the pancreas began by isolating the spleen and ligating the left gastric vessels. The hepatic artery was divided and the left lobe of the pancreas freed taking care to preserve the coeliac artery pedicle and portal vein. The aorta was mobilized sufficiently to allow ligation of all the branches including the renal and superior mesenteric arteries, and particularly the lumbar vessels for one centimeter either side of the coeliac artery. This ensured no leakage from the aorta when the preparation was placed on the perfusion apparatus.

Following complete dissection of the left lobe of the pancreas, the animal was given an intravenous injection of 100 units of sodium heparin in 1 ml of normal saline and the pancreas flushed in-situ with 1 ml of ice-cold saline through the aorta. The perfused organ was then gently removed and stored in saline at 4°C until transplanted into a diabetic animal.

4.4.2 Recipient Procedure - pancreas transplantation

Three weeks following induction of diabetes the blood sugar was measured using a Glucometer (American Scientific Supply, IL.); diabetes was confirmed if the blood sugar remained above 300 mg/dl. The animal was shaved, the abdomen cleaned with either 70% alcohol or povidone iodine, and following a

FIG. 4.1

SCHEMATIC DIAGRAM OF PANCREATIC MICROSURGICAL ANASTOMOSIS

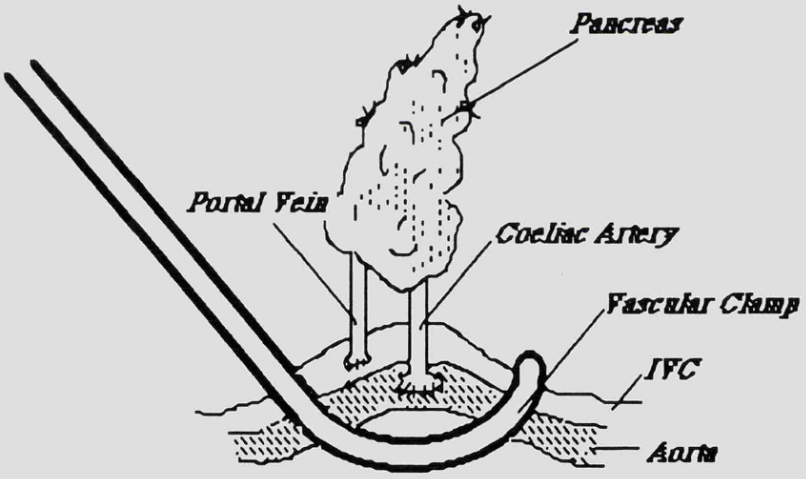

Fig. 4.1 This schematic diagram shows the technique of the vascular anastomosis employed in the pancreatic transplantations. The host vessels were isolated in a vascular clamp. A Carrel patch on the coeliac artery was anastomosed to the aorta using continuous 8.0 proline. The portal vein was anastomosed to the inferior vena cava (IVC) using interrupted sutures. The pancreatic duct was ligated (not shown).

midline incision the aorta and inferior vena cava distal to the renal vessels isolated. A segment of both aorta and vena cava was isolated in a vascular clamp and a small arteriotomy and venotomy cut respectively. A Carrel patch was created on the donor pancreas' coeliac artery and anastomosed to the aorta using 8-0 proline (Davis and Geck, N.Y.) continuous haemostatic sutures. The pancreas' portal vein was anastomosed to the recipient inferior vena cava using an approximating 8-0 proline suture (Diagram 4.1). Once the blood flow to the pancreas was established the pancreas was secured to the posterior abdominal wall with two proline sutures. The pancreatic duct was either ligated or left open to drain freely into the peritoneal cavity. To aid dissection, magnifying Loupes, 2.5x magnifying power, were used; a dissecting microscope was not used in any experiment.

4.5 THE PREPARATION OF PANCREATIC ISLETS

4.5.1 Outline

The first account of successful isolation of pancreatic islets was by Moskalewski, who reported the action of collagenase on the pancreas of guinea pigs (Moskalewski, 1965). Soonafter, Lacy and Kostianovsky modified the technique and reported successful transplantation of rodent islets (Lacy and Kostianovsky, 1967). In 1972, Ballinger and Lacy described the ductal distension of the pancreas prior to collagenase digestion and subsequent transplantation into rats (Ballinger and Lacy, 1972); One further modification has increased the islet yield from whole pancreases in many animal models. Horaguchi and Merrel described the use of continuous infusion of of collagenase solution into the pancreatic duct, which resulted in extremely good isolation of relatively pure islets (Horaguchi and Merrel, 1981). Subsequently, with modifications by Warnock (Warnock *et al,* 1983) and Noel (Noel *et al,* 1982), Alejandro and colleagues have described the use of ductal distension with the inclusion of collagenase in the distending solution (Alejandro *et al,* 1986). They

reported that the use of collagenase with ethylene glycol - bis(β-amino ethyl ether) tetra acetic acid, E.G.T.A., a calcium chelating agent, the numbers of islets isolated from each pancreas were significantly higher (300-400,000 islets from a single canine pancreas) and that they were almost 90% pure. This technique has been employed by others, with improved islet yields from the human pancreas (Gray *et al,* 1984). The intra-ductal collagenase technique has now been applied to rodents with concomitant improvement in islet yield and purity. It is this technique which was used in the experiments for these studies.

4.5.2 Neutral Red

To facilitate identification of the pancreatic islets and to distinguish them from contaminating lymphoid tissue, the islets were stained dark red by injecting the animal with neutral red dye, just prior to the pancreas removal.

In 1911, Bensley perfused animal pancreases with a dilute solution of the dye, neutral red. He described that there was preferential staining of certain cellular components of the pancreas which we now identify as the islets of Langerhans (Bensley, 1911). Since then, many researchers have used this technique to aid identification of pancreatic islets (Bretzel *et al,* 1980; Downing *et al,* 1980). Even though the identification of the pancreatic islets were improved it needed to be proven that the dye caused no functional of immunological impairment to the islets themselves. Indeed, Gray has already addressed this issue (Gray *et al,* 1983). The neutral red was used in all experimental groups.

4.5.3 Procedure for Isolating Pancreatic Islets

The technique used in the following experiments was a modification of the collagenase ductal distension method used by Gray *et al.* The animals were anaesthetised with an intraperitoneal injection of chloral hydrate, 30 mg/kg, and the abdomen washed in 70% ethanol alcohol following shaving. All the instruments used were sterilised in an autoclave, together with the suture

injection of 4 mls of Neutral Red (Sigma Laboratories, St. Louis, MO.) to stain the islets. The liver was then everted cranially over the costal margin and the pancreatic/bile ducts identified. A small haemoclip was placed over the distal duct adjacent to the duodenum and an 18 G Angiocath inserted and secured in the common bile duct with a small silk ligature. The animal was sacrificed by allowing exsanguination through an incision in the aorta and the pancreas immediately distended with the ice-cold collagenase solution. 5 mls of ice-cold Hanks Balanced Salt Solution (HBSS, Gibco) containing 0.7 mg of collagenase, Sigma Type XI (Sigma), and 5 % fetal calf serum (Gibco) were injected into the pancreatic duct. The collagenase was of high activity (1680 units/dry weight) and had low Clostripan, Caseinase and Tryptic activity (0.12, 14 and <0.3 units /dry weight, respectively). The concentration of the collagenase solution was 0.5 mg solid collagenase/ml HBSS. Immediately following ductal distension the pancreas was removed. Care was taken not to enter the substance of the pancreas or damage the delicate pancreatic capsule surrounding the organ in order to avoid leakage of the collagenase solution from the organ. The pancreas was immediately placed in a 15 ml test tube containing 4 mls of warmed (37°C) Hanks Solution (HBBS), and incubated for twenty minutes at 37°C.

At the termination of the incubation period, the excess fluid was discarded from the test tube and the pancreas shaken vigorously for one or two minutes. When visible islets, free of contaminating acinar tissue, could be identified, the digestion was stopped by adding cold (4°C) HBBS and mixing the contents thoroughly. The pancreatic digest was washed three times in HBBS, and finally in HBBS containing 10% fetal calf serum (FCS). This digest contained free isolated islets, together with digested acinar tissue, fibrous and connective tissue, and lymph nodes. The islets were separated from the other debris in the following manner.

The digest was diluted into twelve millilitres of HBBS with 10% FCS and divided into four conical 15 ml test tubes, each therefore, containing 3 mls of pancreatic digest. One ml of Histopaque (Gibco Labs., specific gravity 1.077), a density gradient containing ficoll and iodine-hypaque, was carefully underlaid beneath the digest solution, creating a clear interface. The tubes were centrifuged at 800 G for 10 minutes and the tissue at the interface aspirated and placed in a clean conical 15 ml tube. The pellets, from all four tubes, which mainly contained non-islet tissue, were mixed and placed in another tube. The purity of

the islets from the interface at this stage was only about 70%. There was considerable contamination with fibrous fragments and small lymph nodes. This was subjected to further purification by a second centrifugation over the density gradient.

The tissue at the interface, and the cellular debris were spun over Histopaque for 10 minutes. Any islets freed from the debris were hand picked and added to to yield. More importantly, the tissue at the interface in the first tube, now consisting 95% pure islets was washed in Dubecco's Modified Essential Medium (Gibco, DMEM), containing 10% FCS and kept on ice. The islets were transferred to a Petri dish and carefully hand picked under a dissecting microscope, and stored on ice before further manipulation. Only pure islets were chosen and in most cases the islets were handpicked twice. In this way, although 500 - 700 islets were obtained from each animal, only 300 - 400 islets were considered pure or large enough for immunological manipulation or transplanation.

Experiments involving the transplantation of pancreatic islets and the assessment of their viability and function following these isolation procedures, will be discussed in Chapter 9.

4.6 MONOCLONAL ANTIBODIES

4.6.1 Introduction

The rat appears to express Class II MHC antigens of both I-A and I-E type homologous to the mouse and, indeed, there appears to be a high level of cross reactivity between the monomorphic determinants of mouse and rat I-A and mouse and rat I-E (Fukumoto *et al,* 1982). Consequently, many monoclonal antibodies made in mice or reactive against mouse determinants could be used to label rat tissue.

4.6.2 OX6

OX6 (Seralab, Accurate Chemicals, New York, N.Y.), is an IgG_1,

kappa mouse antibody which recognizes a monomorphic rat Class II antigen determinant. This MAb crossreacts with a mouse Ia determinant which maps to the I-A subregion; it reacts with spleen cells from mice of the H-2^s haplotype, which are known to lack I-E expression, suggesting that it truly recognizes the I-A homologue in the rat.

4.6.3 14.4.4. and ISCR3

The cell lines for both these MAb's were a kind gift from Dr. David Sachs at the National Institute of Health, Bethesda, Maryland. The monoclonal antibodies were produced and purified by Marguerite Buckingham. The hybridoma cells were injected into ascites induced nude mice and antibody enriched ascitic fluid tapped at three weeks post injection. Both these MAb's are IgG-2a, kappa MAb's recognizing an I-E^k determinant which corresponds to the previously described crossreactive Ia serum specificity present only on cells of the I-E expressing strains of mice. These MAb's were produced by C3H.SW anti-C3H, and ATH anti-ATL immunisations, respectively. Both monoclonals cross react with a monomorphic determinant on rat splenocytes of all rat strains tested; immunoprecipitation of rat lymphocyte membranes with anti-Ia positive sera yields two protein bands of molecular weight similar to mouse Ia alpha and beta chains and presume to be the I-E homologue (Wettstein *et al,* 1981).

Therefore, even though OX6 , 14.4.4. and ISCR3 are Class II reactive in the rat, their actual Class II specificities are different. The rat MHC region has been mapped, such that the regions RT1- *A, C,* and *E* code for Class I antigens, and RT1- *B* and *D* code for Class II antigens. In the mouse, the H-2 region has been extensively mapped such that the Class II region has been subdivided into I-E and I-A. It would appear therefore that OX6 is reactive against one epitope on the Class II antigen in the rat, analogous to the I-A^k region in the mouse, and both 14.4.4. and ISCR3 are reactive against another Class II determinant, analogous to the I-E^k region in the mouse. It would seem logical, that in order to obtain optimal lysis of cells by complement (C'), then the more antigenic determinants labelled with the C'-fixing antibody, the better chance of lysis. Perhaps the use of a combination of monoclonal antibodies would increase the chance of dendritic cell depletion. Furthermore, if the immunological sequalae of pretreating tissues

depended on the 'masking' effect of the MAb's, then using a cocktail of MAb's reactive against several differing Class II determinants on the cell surface, would mask more antigenic determinants and enhance this phenomenon.

CHAPTER 5

EX VIVO PERFUSION

5.1 INTRODUCTION

Continuous perfusion of renal allografts has been a viable and certainly a practical solution to organ preservation for almost two decades (Pegg and Jacobsen, 1979). In the past, continuous perfusion has permitted longer periods of storage than non-perfusion methods, but with the development of better preservation fluids, together with an increased understanding of hypothermic physiology, prolonged cold storage of the unperfused organ is proving successful (Opelz and Terasaki, 1982; Collins, 1985). However, whichever method of preservation is employed, there are limiting factors to either technique. Abouna and colleagues emphasised the possible importance of ammonia toxicity (Abouna *et al,* 1972), but others have reported that during cold storage or cold perfusion, ammonia does not accumumulate (Cunarro *et al,* 1976). The advantages of perfusion preservation, include possible elimination of vasospasm and washing out of blood components, including ATP-depleted deformed red blood cells (Weed *et al,* 1969), prediction of viability by perfusion flow and pressure, and perhaps, prolonged perfusion may reduce the actual immunogenicity of the organ itself (Lafferty *et al,* 1976). However, even though the apparent advantages of continuous perfusion, such as control of nutrients, osmotic and oncotic pressures, oxygen pressure, redox potential and pH may be equalled or at least not significantly affected by cold storage, there is one further application which is not applicable to that of simple cold storage; that is, the *immunoalteration* of the organ during preservation.

Considerable discussion has taken place as to which method, either simple cold storage or machine preservation, is better for kidney preservation in humans. It has been reported recently that renal perfusion by mechanical devices is superior if extended preservation times are required or if the kidneys have been exposed to significant periods of warm ischaemia (Belzer, 1985).

Whether pulsatile machine perfusion techniques can also be applied to

pancreas preservation depends on the specific characteristics of the pancreas, which generally requires a relatively low blood flow rate through it's vasculature, in contrast to the kidney. Also, unlike the kidney, in the pancreas, prolonged periods of machine preservation over 24 hours has not been reported, though successful cold storage of the pancreas up to 72 hours has been achieved (Belzer *et al,* 1985). Various organ preservation machines are commercially available for application to the pancreas including the Gambro machine (Brynger, 1975), the Belzer machine (De Gruyl *et al,* 1977) and the Mox-100-machine (Toledo-Pereyra *et al,* 1979). For hypothermic pancreas preservation these machines were filled with different perfusates. Graft oedema developed in most cases. Generally, the preservation of the pancreas by mechanical perfusion is not as successful as simple flushing and cold storage techniques. Indeed, in one report, not only were the results inferior, but also many adjustments had to be made to the perfusion apparatus and perfusion characteristics to achieve any function following perfusion (Florek *et al,* 1988). The flow rate was reduced to below 10 mls/min and the osmolality of the perfusate increased to 470 mOsm/kg to avoid graft oedema. Despite these changes the preservation failure rate after 24 - 48 hours perfusion was still 30 - 40%.

The experiments designed in this dissertation, for the *ex vivo* perfusion of the rat pancreas,were intended to study the effects of perfusion for relatively short periods. Long term preservation of the pancreases by the proposed regimen was not attempted. The perfusion parameters were therefore adjusted such that the effects of MAb manipulation to the intact and isolated organ could be optimised and determined.

A perfusion circuit was developed to investigate the role of monoclonal antibody manipulation of organ allografts in an ex-vivo normothermic environment. The rationale in developing this circuit was to extend previous reports by others (Asano *et al,* 1987; Faustman *et al,* 1981, 1984) that pretreating tissues with anti-Class II monoclonal antibodies reduces graft immunogenicity. Application to an ex vivo perfusing system would allow high doses of the monoclonal agents to be used such that maximum exposure (prolonged perfusion) in a normothermic environment would optimise complement-dependent lysis of the Class II MHC antigen bearing cells.

5.2 DEVELOPMENT OF THE EX VIVO PERFUSION CIRCUIT

Utilising silicon tubing and a non-peristaltic roller pump, a basic circuit was developed which allowed the continuous perfusion of isolated rat organs. The rate of perfusion was controlled by the pump flow rate and the diameter of the tubing. For complement mediated antibody lysis of target cells contained within the pancreas, the system had to function at normothermia (37°). For this, a small hollow glass chamber was designed as a heat exchanger in which the organ was placed. The glass chamber was attached to a water bath and an independent water pump to allow heated water to circulate through it. The temperature of the perfusate was monitored by a thermometer in the perfusate and the temperature of the circulating water adjusted as necessary to maintain a perfusate temperature of 37°C. This added further difficulies.

Previous studies had shown that rat pancreases, in contrast to rat kidneys, could withstand warm ischaemia for up to one hour (Schulak *et al,* 1983). However, longer periods were not tolerated. To increase the length of time the organs could be exposed to anti-Class II MAb's, it was necessary to develop techniques which would allow many hours of normothermic ex vivo perfusion. Consequently, the perfusate had to transport and deliver oxygen to the rat pancreas. The perfusate had to contain haemoglobin and consequently be oxygenated. This was ultimately achieved by inserting a single set of multiple hollow fibres, 80-100 microns internal diameter, similar to those used for renal haemodialysis, into the perfusion circuit and placing this 'membrane' in a fleaker system into which 100% oxygen was delivered.

5.3 THE APPARATUS (See Fig. 5.1; and Plates 5.1, 5.2, 5.3)

The final apparatus thus consisted of a non-peristaltic pump (Gibco, Grand Island, New York), a 37°C glass warming chamber and a hollow fibre membrane oxygenator (Spectrum Laboratories, Los Angeles, CA.). The perfusion tubing itself was silicon plastic (Travenol, Deerfield, Illinois) and the organ receptical fashioned from the lower half of a 50 ml Falcon tube. A 40 μm

FIG. 5.1

SCHEMATIC DIAGRAM OF PERFUSION CIRCUIT

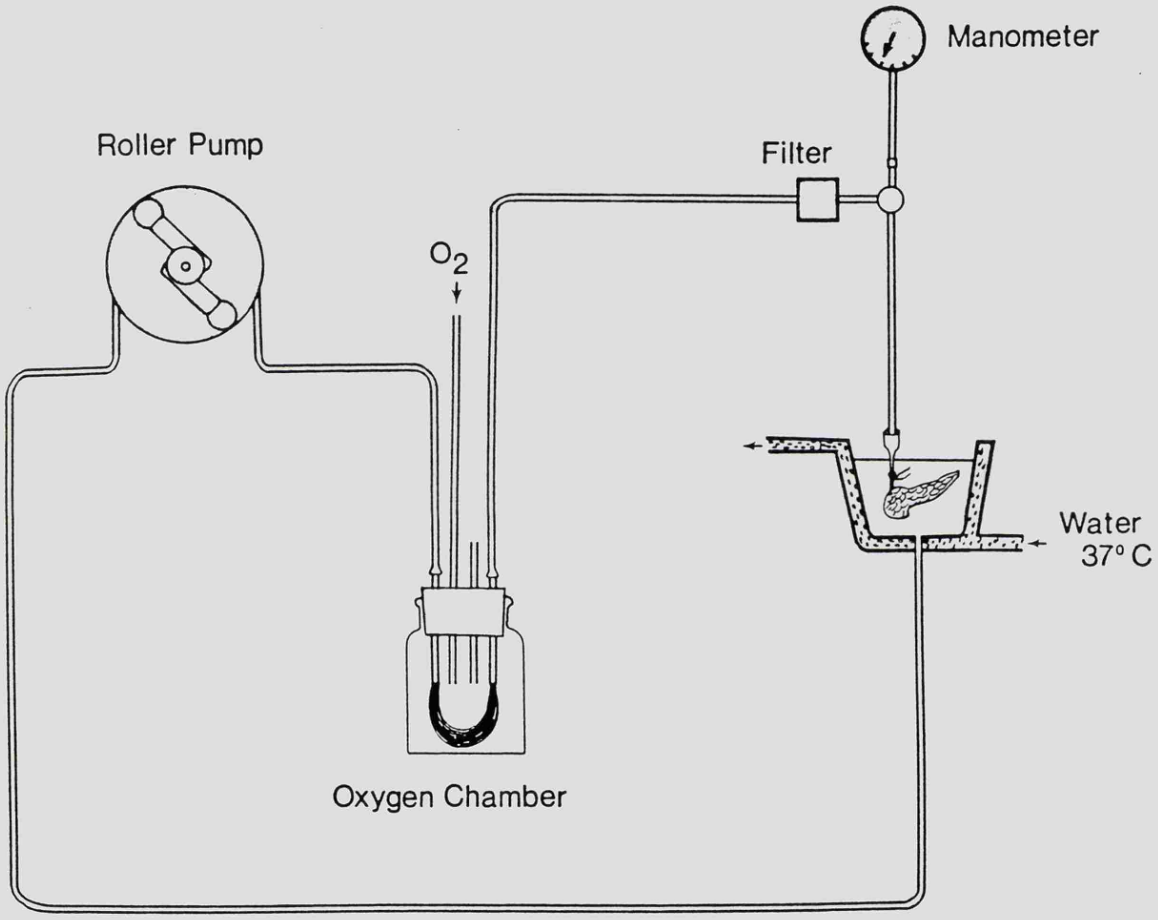

Fig. 5.1 Schematic diagram of the Ex Vivo Perfusion circuit developed for the perfusion of rat pancreases. The pancreas is perfused with blood, containing monoclonal antibodies, which is recycled through the circuit by means of a roller pump. The perfusate is filtered before entering the pancreas and the pressure within the aortic cannula monitired with a manometer. The system is kept at 37°C and the perfusate oxygenated in the oxygen chamber.

PLATE 5.1

PHOTOGRAPH OF PERFUSION APPARATUS

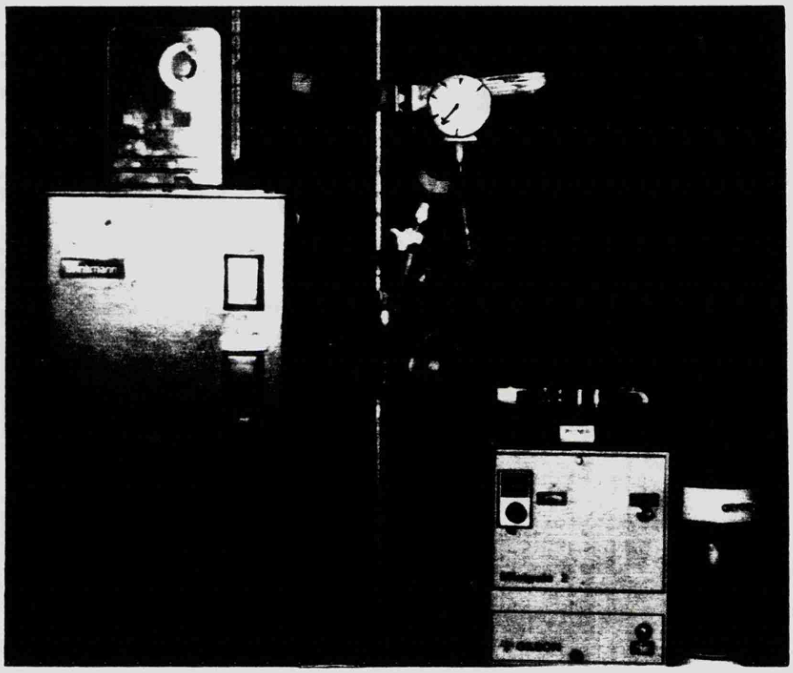

Plate 5.1 A photograph of the actual perfusion circuit, showing the perfusion chamber at the centre. The water bath for heating the perfusate is situated on the left. The blood was pumped through the circuit using a non-peristaltic Gilson pump, and oxygenated via a dialysis membrane (bottom right). The pressure within the system was continually monitored with a manometer (top).

PLATE 5.2

PHOTOGRAPH OF THE PERFUSION KIT BEFORE ASSEMBLY

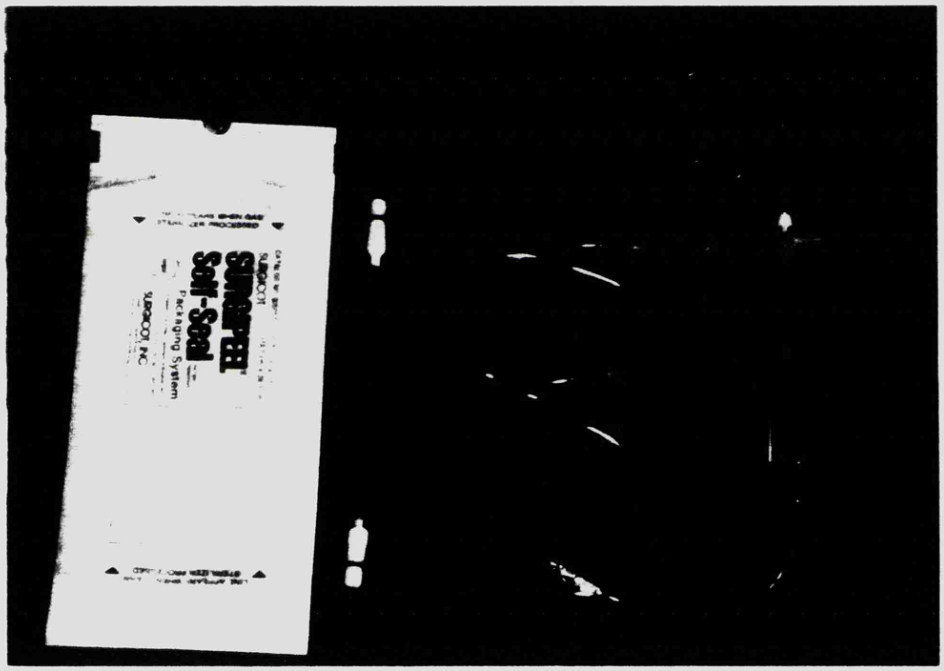

Plate 5.2 Photograph showing the basic kit, prior to assembly, for perfusing rat pancreases. The circuit consisted of silicon tubing, with a chamber in which the organ was placed, made from the lower half of a large Falcon test tube (right). The perfusate was oxygenated through a membrane made of dialysis tubing (left). The kits were made up in advance and gas sterilised in separate packets (far left).

PLATE 5.3

CLOSE UP PHOTOGRAPH OF A PANCREAS BEING PERFUSED ON THE EX VIVO CIRCUIT

Plate 5.3 Photograph showing a close up view of a rat pancreas being perfused with blood on the ex vivo perfusion circuit. The pancreas is perfused with blood via the coeliac artery, by means of a cannula in the aorta.

In this photograph the pancreas has been lifted out of the perfusion chamber to demonstrate the blood exuding from the portal vein (held in the forceps on the left). The pancreas was usually placed in the chamber and bathed by it's own perfusate. Note that the pancreas is neither oedematous nor ischaemic. Pancreases can be perfused in this way for up to six hours without significant functional impairment.

filter (Millipore, Harrow, U.K.) was included in the circuit to filter any debris or cellular clumping which may have occurred. The pancreas was isolated on it's coeliac artery attached to its aorta, and a 22 French gauge Angiocath placed in the aorta and secured with a ligature. Finally, a green Butterfly, 23 French gauge, was placed in the Angiocath and attached to a pressure manometer which permitted continuous reading of the pressure actually within the aortic cannula and the pancreas itself. The circuit was primed with 7.5 mls of heparinised, buffy coat depleted blood and diluted with Plasmalyte (Travenol, Deerfield, Il.) to a final haematocrit of 20%. Fifty micrograms of verapamil were added to the perfusate to allow vasodilatation of the small capillaries within the pancreas and thus optimise the delivery of the monoclonal antibodies to all parts of the organ. The monoclonal antibodies were added to the perfusate to a final concentration of 20 μg/ml perfusate. For the pancreas the optimal pressure was determined to be 40 mm Hg with a flow of 1.75 mls/minute (see below). The temperature was checked intermittently throughout the procedure to maintain normothermic conditions.

5.4 OPTIMAL CONDITIONS FOR THE EX VIVO PERFUSION OF THE RAT PANCREAS

Initial conditions which were investigated concerning ex-vivo rat pancreas perfusion included the viscosity of the blood perfusate, the pressure within the system and the length of time a pancreas could be perfused ex-vivo, using this artificial oxygenating, haemoperfusion circuit and still remain functional following allograft transplantation. Thus, the pressure-flow relationship within the perfusion circuit had to be carefully assessed.

Initially, pancreases perfused with whole blood alone for only one hour did not survive or function following transplantation into syngeneic diabetic recipients. The pressure within the aorta rose to pressures exceeding 80 mm Hg and the pancreases became oedematous and haemorrhagic. The viscosity of the blood was too high for optimal flow conditions on the circuit. The blood was diluted in normal saline to a haematocrit of 50% and 20%. Although pressures in the system were maintained around 40 mm Hg the pancreases were still not

FIG 5.2

SURVIVAL OF PANCREASES PERFUSED AT VARYING FLOW RATES

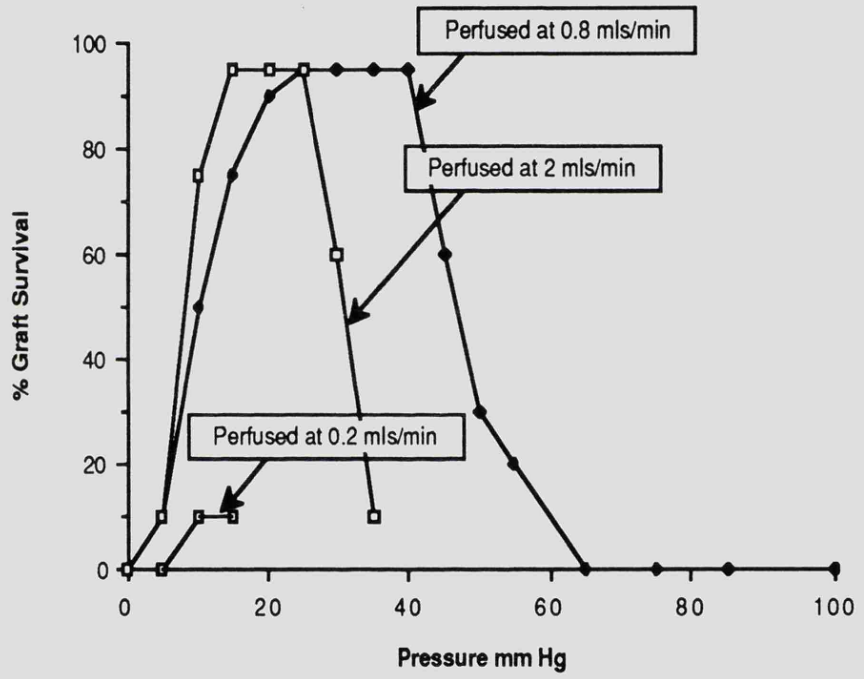

FIG. 5.2 Graph showing the effect of varying the flow rate of the perfusate on the perfusion pressure within the graft and on the survival of syngeneic pancreas transplants following one hour ex-vivo perfusion.

Flow rates below 0.2 ml/min and above 2 ml/min were not associated with good pancreatic viability. The survival of allografts was particularly dependent on perfusion pressure too. Pressures below 15 mm Hg and above 50 mm Hg were associated with low graft survival. The best survival was achieved when the perfusion pressure was between 20 and 40 mm Hg.

TABLE 5.1

SURVIVAL OF PANCREASES PERFUSED WITH OR WITHOUT RABBIT COMPLEMENT

Group	Perfusion Period	Additive	Graft survival (days)	Mean ± SD (days)
1.	no perfusion *(n = 12)*	none	9, 10(x5), 11(x4), 12(x2)	10.9 ± 0.9
6.	3 hours *(n = 10)*	C' alone	10 (x4), 11, 11, 12, 12, 14, 14	11.4 ± 1.6
7.	3 hours *(n = 7)*	none	10, 10, 11, 11, 12, 12, 15	11.5 ± 1.7

Table 5.1 Comparison of the control groups 1, 6 and 7. This table shows that there was no significant difference in allograft survival when pancreases were perfused with complement (C') (group 6) or without complement (group 7) for three hours, when compared to untreated pancreas transplants (group 1). ($p>0.5$ when each group was compared to each of the others).

TABLE 5.2

SURVIVAL OF PANCREATIC ALLOGRAFTS FOLLOWING DIFFERENT PERFUSION PERIODS

Group	Perfusion Period	Additive	Graft survival (days)	Mean ± SD (days)
1.	no perfusion *(n = 12)*	none	9, 10(x5), 11(x4), 12(x2)	10.9 ± 0.9
2.	no perfusion flush only *(n = 4)*	14.4.4. and OX6	9, 10, 10, 11	10.0 ± 0.8
3.	1 hour *(n = 7)*	14.4.4. and OX6 and C'	10, 11, 11, 12, 13, 13, 14	12.0 ± 1.4
4.	2 hours *(n = 6)*	14.4.4 and C'	11, 11, 13, 13, 15, 15	13.0 ± 1.8
5.	3 hours *(n = 14)*	14.4.4. and OX6 and C'	11, 13, 14 (x3), 16 (x6) 17, 23, 23	16.1 ± 3.3
6.	3 hours *(n = 10)*	C' alone	10 (x4), 11, 11, 12, 12, 14, 14	11.4 ± 1.6

Table 5.2 Survival of $LDAF_1$ pancreatic allografts transplanted into diabetic DA recipients following either ex vivo perfusion with or without anti-Class II MAb's (14.4.4. and OX6) included in the perfusate together with rabbit complement (C'). Group 1, for comparison were straight transplants that had not been perfused, and in group 2, the pancreases were simply flushed with 14.4.4. and OX6 just prior to transplantation. The prolongations of allograft survival achieved following 2 hour (Group 4) and 3 hour (Group 5) perfusion with MAb and C' were significant when compared to perfusion with C' alone (Group 6) ($p<0.005$, $p<0.0001$ respectively). A 1 hour perfusion also produced a significant prolongation in allograft survival ($p<0.003$).

viable following transplantaion. The sodium levels were thought to be high and non-physiological, and indeed when measured were between 160 and 170 mEq/l. The blood was then diluted with Plasmalyte (Travenol, Deerfield, IL.), instead of normal saline. Pancreases perfused with this more physiological solution now survived following transplantation after an hour of ex vivo perfusion.

The flow rate was reduced below 3 mls per minute, as flows above this level produced severe oedema in the pancreases and were associated with petechial haemorrhages, occasional rupture of the larger vessels and a high percentage of primary non-function of the pancreas. Flow rates below 0.2 - 0.3 mls/min. were not associated with much swelling, but following transplantation the high incidence of graft failure was thought to be due to relative tissue hypoxia. Increasing the flow to 0.8 - 0.9 ml/min. at the 30-40 mm. Hg. pressure range certainly improved survival, but the addition of the vasodilatory calcium channel blocker, verapamil, at a concentration of 2.5 μg/ml, allowed further increase in flow up to 1.75 ml/min. whilst maintaining the perfusion pressure of 30 - 40 mm.Hg. Under these conditions, ex-vivo normothermic haemoperfusion could be extended up to 4 -5 hours before significant deterioration in graft function occurred. At these perfusion parameters, following three hours of perfusion, pancreases transplanted into syngeneic, diabetic recipients maintained long term normoglycaemia (Fig 5.2).

5.5 RESULTS

5.5.1 The Effect of Ex Vivo Perfusion on the Viablity of Rat Pancreas Allografts

In the initial series of experiments, $LDAF_1$ pancreases were perfused for varying periods of time on the perfusion circuit and transplanted into diabetic syngeneic $LDAF_1$ recipients. Within twelve hours following perfusion and transplantation, the recipient animals became normoglycaemic. In one series, 6 pancreases (100%) reversed experimentally induced diabetes for more than 3 months. At that time two of the animals became hyperglycaemic and autopsy revealed almost complete resorption of the pancreatic graft. Histological sections

showed no evidence of viable pancreatic tissue in the donor site and demonstrated only fibrous stroma. These findings, however, were similar to those found in experimental groups in which pancreas grafts were not perfused at all. The atrophy and resorption of the pancreatic grafts after several months were common findings in most segmental grafts in which the pancreatic duct was ligated. Similar findings have been reported by others (Pound and Walker, 1981). This relatively late finding was of no consequence as only short term function was of interest in the allogeneic model. The other 4 sygeneic transplants functioned for six months at which time the residual pancreas grafts were removed. Two of the animals died during this procedure due to haemorrhage, while the two remaining became hyperglycaemic within 12 hours. This confirmed that the pancreatic transplant was responsible for the euglycaemic state, and that the native pancreas had not recovered its function following streptozotocin-induced damage to the islets.

Thus, a period of 3 hours ex vivo perfusion did not significantly damage the viability of pancreatic grafts, $LDAF_1$ pancreatic segments were perfused for 3 hours and transplanted into allogeneic diabetic DA recipients. In the control group (Group 7), pancreases were perfused for three hours with no additives to the perfusate, and transplanted into diabetic DA hosts (see Table 5.1). All the pancreases functioned well and reversed diabetes for between 10 and 15 days until allograft rejection, indicated by hyperglcaemia, occurred (mean survival 11.5 ± 1.7 days, $n = 7$). The mean survival was not statistically significant from untreated unperfused controls which rejected between 9 and 12 days (Group 1, mean graft survival 10.58 ± 0.9 days, $n = 12$, $p > 0.01$) (Fig. 5.3). Furthermore, the addition of 1 ml of rabbit complement (C'), did not affect allograft survival significantly. When C' was added to the perfusate of pancreases perfused for 3 hours, the mean survival of these allografts was 11.4 ± 1.6 days, with a range from 10 to 14 days (Group 6, $n = 10$, $p > 0.01$).

FIG 5.3

SURVIVAL OF PANCREAS ALLOGRAFTS PERFUSED WITH AND WITHOUT RABBIT COMPLEMENT

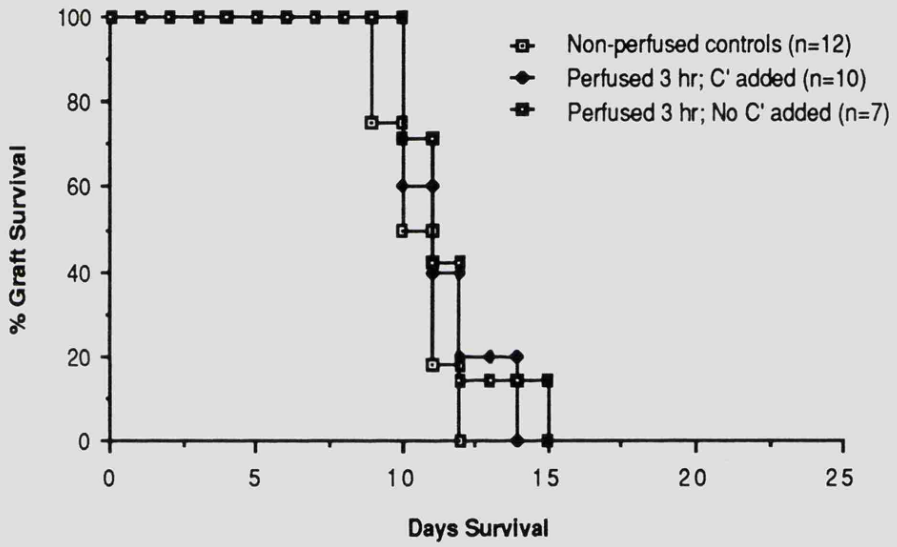

Fig. 5.6 The effect of 3 hrs ex vivo perfusion, with and without the addition of rabbit complement (C'), on the survival of $LDAF_1$ to DA pancreas allografts.

This graph confirms that a 3 hr perfusion period does not significantly effect pancreatic allograft survival. Mean allograft survival of untreated pancreases was 10.9 ± 0.9 days, n=12. When pancreases were perfused for three hours their mean survival was 11.4 ± 1.6 days, n=10 ($p>0.5$, not significant). Similarly, the addition of C' did not effect the allograft survival (11.5 ± 1.7, n=7, $p>0.5$).

5.5.2 *The Effect of Flushing Pancreatic Grafts with Anti-Class II MAb's on Allograft Survival.*

A series of pancreases were removed from $LDAF_1$ donors and flushed with 1 ml of ice-cold saline containing 40 λ of 14.4.4. and 40 λ of OX6 ascites. The grafts were kept at 4°C for 30 minutes prior to transplantation into DA recipients. These pancreases reversed the experimentally induced diabetes for between 9 and 10 days (Group 2, mean 10.0 ± 0.9 days, n = 4). This was not significantly different from any of the control groups, 1, 6 or 7 ($p > 0.2$), in which no MAb's were used (see Table 5.2 and Fig. 5.4).

5.5.3 *The Effect of Perfusing Pancreas Grafts for 1, 2, or 3 hours with Anti-Class II MAbs on Allograft Survival*

In this series of experiments, the pancreases were exposed to both 14.4.4. and OX6, for either 1, 2 or 3 hours, in the presence of rabbit complement, on the ex vivo circuit. Pancreases perfused for 1 hour only, had the C' added after 20 minutes to allow sufficient time for binding of the MAb's and allow sufficient time for complement lysis of target cells. Pancreases perfused for periods longer than 1 hour had the C' added at 60 minutes. At this time maximum labelling of the Class II positive cells within the pancreas has been acheived. This is discussed in the following chapter (see Table 5.2 and Fig. 5.1).

Pancreases perfused with anti-Class II MAb and C', upon transplantation, reversed diabetes in allogeneic recipients for periods ranging from 10 - 14 days with a mean allograft survival of 12.0 ± 1.4 days (Group 3, n = 7) (see Table 5.1 and Figs 5.5 - 5.8). Similarly, pancreases perfused for 2 hours had survival times between 11 and 15 days with a mean survival of 13.0 ± 1.8 days (Group 4, n = 6).

FIG. 5.4

GRAPH COMPARING FLUSHING WITH ANTI-CLASS II MONOCLONAL ANTIBODIES ON THE SURVIVAL OF PANCREAS ALLOGRAFTS

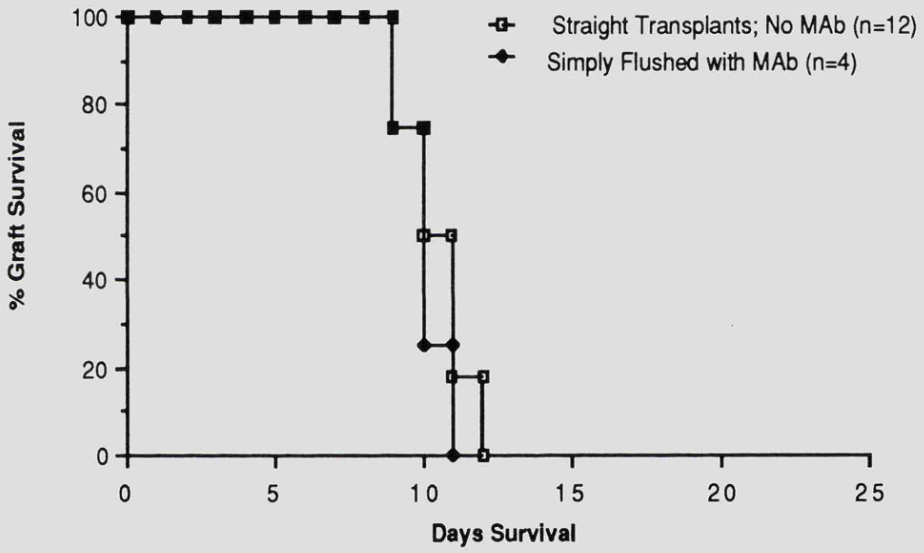

Fig. 5.4 The effect of flushing rat pancreases with anti-Class II MAb (14.4.4. and OX6) on allograft survival.

This graph shows that there was no prolongation of allograft survival when $LDAF_1$ pancreases were simply flushed with MAb, compared to straight transplantation alone. The mean allograft survival of pancreases flushed with MAb was 10.0 ± 0.8 days, n=4. This was not significantly different from untreated controls, mean allograft survival 10.9 ± 0.9 days, n=12.

FIG. 5.5

GRAPH COMPARING THE EFFECT OF PERFUSING THE PANCREAS FOR 1, 2, OR 3 HOURS WITH MAb UPON ALLOGRAFT SURVIVAL

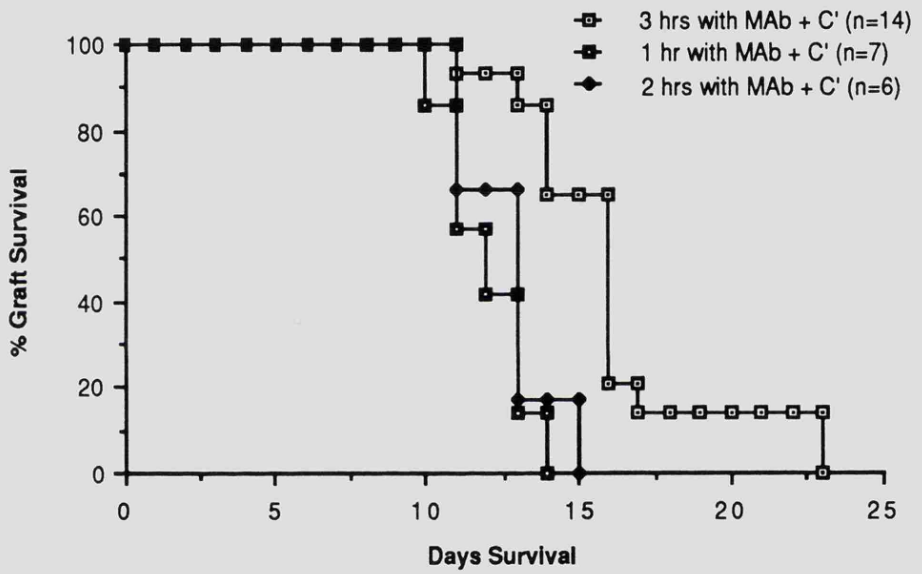

Fig. 5.5 The effect of ex vivo perfusion on the survival of pancreas allografts in the $LDAF_1$ to DA strain combination. This graph compares the survival of pancreases perfused on the ex vivo circuit for 1, 2 or 3 hours with monoclonal antibodies (MAb), 14.4.4. and OX6, and rabbit complement (C'), included in the perfusate. There was a significant prolongation of allograft survival in the 3 hr perfusion group when compared to the one hour perfusion group ($p<0.005$). However, even though there was a prolongation of graft survival when the 3 hr group was compared to the 2 hr group, it was not significantly different ($p>0.2$).

FIG. 5.6

GRAPH COMPARING ALLOGRAFT SURVIVAL OF PANCREASES PERFUSED WITH MAb AND COMPLEMENT FOR ONE OR THREE HOURS

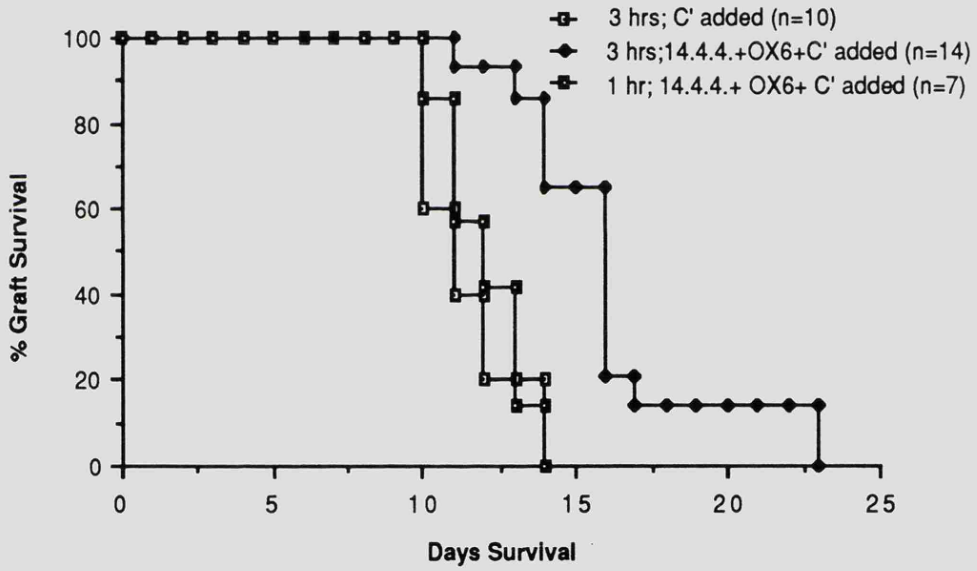

Fig 5.6 The effect on the allograft survival of $LDAF_1$ to DA rat pancreases, when perfused on the ex vivo circuit for 1 or 3 hrs with anti-Class II monoclonal antibodies (14.4.4. and OX6) and rabbit complement added to the perfusate. Pancreases in the control group were perfused for 3 hrs with only C' added to the perfusate.

This graph demonstrates that a prolonged exposure to MAb and C' results in significant allograft survival when the pancreases are perfused with MAb and C' for 3 hrs compared to 1 hr ($p<0.001$) or compared with a 3 hr perfusion with C' alone ($p<0.0001$).

FIG. 5.7

GRAPH CORRELATING THE PROLONGATION OF ALLOGRAFT SURVIVAL TO THE PERFUSION PERIOD

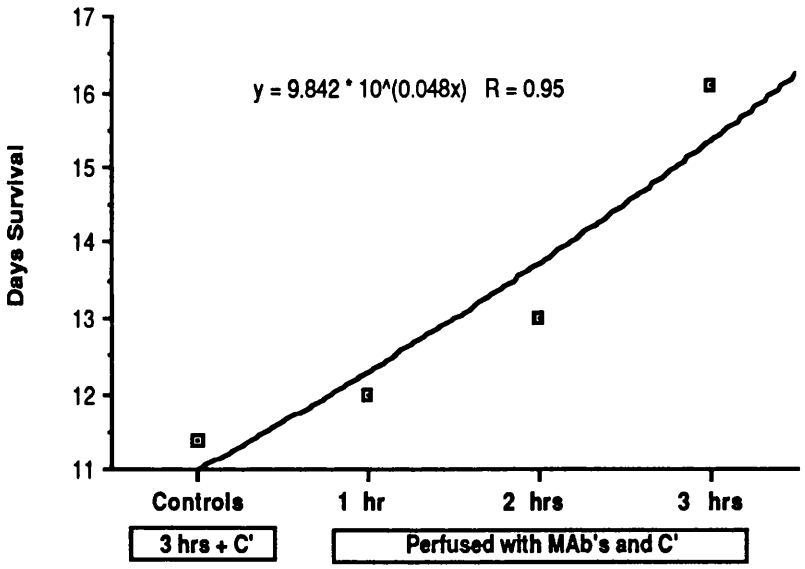

Fig. 5.7 This graph represents the prolongation of survival when $LDAF_1$ pancreas allografts were transplanted into diabetic DA recipients, following 1, 2, or 3 hours ex vivo perfusion with anti-Class II monoclonal antibody (MAb) and rabbit complement (C').

It can be seen that there was a significant increase in the survival time, which is correlated to the length of the perfusion period. The pancreases exposed to the MAb's and C' for 3 hours had an increase in mean allograft survival of 5 days, compared to allografts perfused with C' alone for 3 hours ($p<0.0001$).

FIG. 5.8

BAR GRAPH COMPARING THE INCREASE IN ALLOGRAFT SURVIVAL TIME FOLLOWING INCREASING PERIODS OF PERFUSION

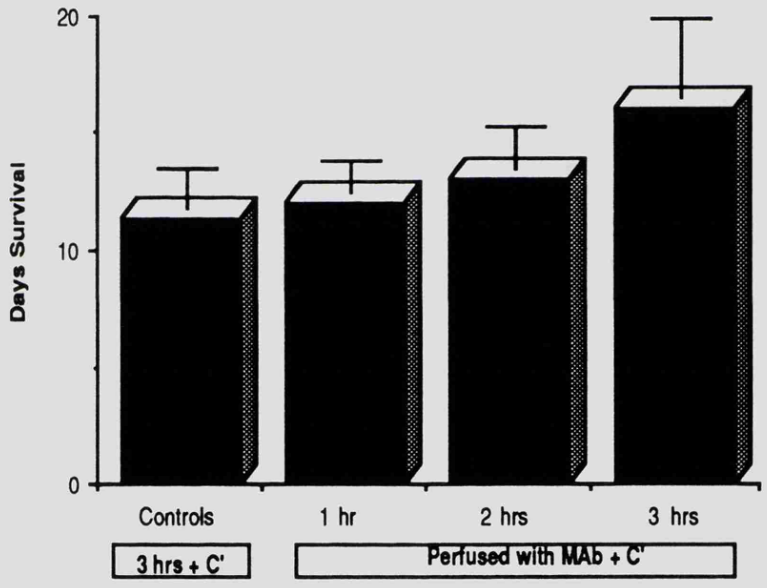

Fig. 5.7 This bar graph represents the prolongation of survival when $LDAF_1$ pancreas allografts were transplanted into diabetic DA recipients, following 1, 2, or 3 hours ex vivo perfusion with anti-Class II monoclonal antibody (MAb) and rabbit complement (C'). It can be seen that there is an increase in the survival time which relates to the length of the perfusion period. The pancreases exposed to the MAb's and C' for 3 hours had an increase in mean allograft survival of 5 days, compared to allografts perfused with C' alone for 3 hours ($p<0.0001$).

Finally, pancreatic segments perfused for 3 hours with 14.4.4. and OX6, survived between 11 and 23 days, with a mean survival of 16.1 ± 3.3 days (Group 5, n = 14). The survival of all these groups were statistically compared to each other and each with control group (Group 6) in which pancreases were perfused for 3 hours with C' alone and no monoclonal antibody (see Table 5.1). There was a significant prolongation of allograft survival when $LDAF_1$ pancreases were perfused with anti-Class II MAb's and C' for 3 hours (Group 5, $p < 0.0001$), 2 hours (Group 4, $p < 0.005$), and even a 1 hour perfusion (Group 3, $p < 0.003$), when compared to the control group. Furthermore, a 3 hour perfusion resulted in a significantly longer allograft survival when compared to a 1 hour perfusion (Group 5 vs. Group 3, $p<0.0006$), but not a two hour perfusion period (Group 5 vs. Group 4, $p > 0.2$).

5.5.4 The Effect of the Addition of Complement to the Perfusate on the Survival of Pancreatic Allografts Perfused with MAb.

This experiment was to determine if the addition of exogenous lypholysed rabbit complement was necessary in order to achieve optimal allograft prolongation following a period of ex vivo perfusion. The serum used to prime the perfusion circuit was of rat origin, a source not rich in complement activity. If the reduction of graft immunogenicity was dependent on the depletion (lysis) of the Class II bearing cells within the pancreas, then the addition of exogenous complement should enhance this result. The labelling of all the Class II positive cells within the pancreas during perfusion with the anti-Class II monoclonal antibodies was established (see Chapter 6). The rabbit complement was absorbed sequentially on rat lymphocytes and splenocytes, to remove preformed antibodies.

This experiment was designed to establish whether complement -dependent lysis of the Ia positive cells in perfused pancreases would have significantly better effects than a 'blocking' effect of the MAb bound to the Class II antigen expressing cells within the graft without actual lysis of these cells by

FIG. 5.9

THE EFFECT OF ADDING COMPLEMENT TO THE PERFUSATE ON THE PROLONGATION OF ALLOGRAFT SURVIVAL OF PANCREASES FOLLOWING PERFUSION WITH MAb.

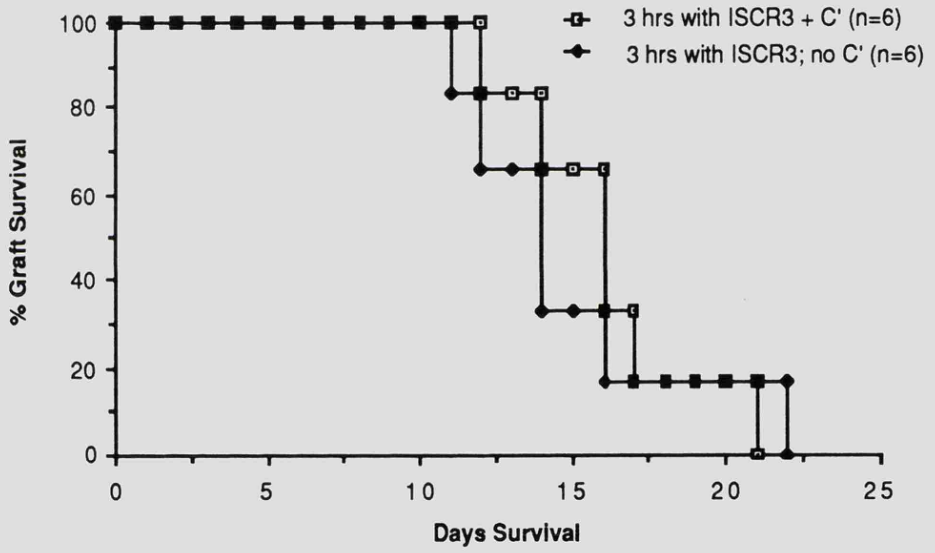

Fig 5.9 The effect of the addition of rabbit complement (C') on the allograft survival of rat pancreases perfused for 3 hrs with the anti-Class II MAb ISCR3.

This graph indicates there is no increase in allograft survival when 1 ml of C' was added to the perfusate. Pancreases perfused with MAb alone reversed diabetes for 14.8 ± 3.9 days, n=6. This effect was not significantly increased when pancreases were perfused for 3 hours with MAb and additional C' (16.0 ± 3 days, n=6, $p>0.1$).

FIG. 5.10

COMPARISON BETWEEN TWO DIFFERENT ANTI-CLASS II MAb'S ON THE ALLOGRAFT SURVIVAL OF PERFUSED PANCREASES

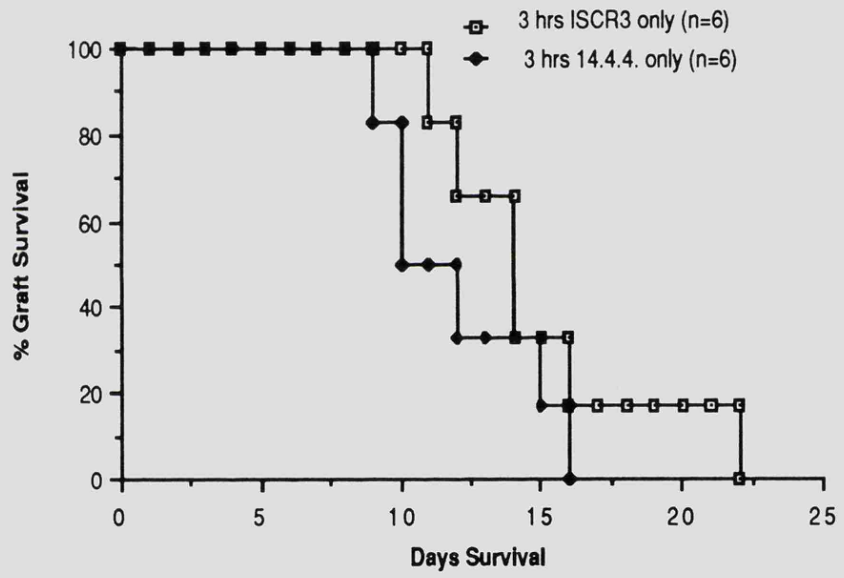

Fig. 5.10 The effect upon allograft survival of perfusing $LDAF_1$ pancreases with two different anti-Class II monoclonal antibodies, 14.4.4. or ISCR3.

There was a significant prolongation of allograft survival when both groups were compared to control groups ($p<0.001$). The mean survival of pancreases perfused with 14.4.4. was 12 ± 2.9 days, n= 6; those pancreases perfused with ISCR3 alone had a mean survival 14.8 ± 3.9, n=6.

complement-antibody destruction.

In other words, if the immunoalteration of the pancreas depended on the depletion of the Class II positive cells by the binding of anti-Class II antibodies and subsequent complement lysis of these cells, the addition of C' should improve allograft survival. Because *in vitro* studies in our laboratory verified that the MAb OX6 did not fix complement (data not shown, complement lysis assay performed by Marguerite Buckingham), and that the MAb ISCR3 lysed 100% of Class II positive spleen cells in the presence of C', OX6 was not included in this study.

When $LDAF_1$ rat pancreases were perfused with ISCR3 and 1 ml of rabbit complement for 3 hours and transplanted into diabetic DA recipients, the mean allograft survival was 16.0 ± 3 days (Group 13, n = 6). When complement was omitted from the perfusate, and pancreases were perfused with ISCR3 alone, the mean allograft survival was 14.8 ± 3.9 days (Group 11, n = 6). These two survival means were not statistically significant from each other ($p > 0.1$). The allograft prolongations achieved in both groups, however, were significantly different from control groups in which pancreases were perfused with no anti-Class MAb's, with or without C' ($p<0.0001$). See Table 5.3 and Fig 5.9.

5.5.5 Comparison between 14.4.4. and ISCR3 Anti-Class II MAb's on the Prolongation of Allograft Survival.

The effects of using two different anti-Class II MAb's was determined. Both 14.4.4 and ISCR3 are reactive against monomorphic rat Class II determinants, analogous to the $I\text{-}E^k$ haplotype mapped on the H-2 region of the mouse, but are from two separate cell lines. Both lyse $LDAF_1$ splenic lymphocytes bearing Ia antigens in the presence of rabbit complement.

The effects on allograft survival by prolonged ex vivo perfusion were compared and shown in Fig. 5.10. Pancreases perfused with 14.4.4 alone reversed hyperglycaemia for between 9 and 16 days with a mean survival of 12 ± 2.9 days (Group 14, n = 6). Those allografts perfused with ISCR3 alone functioned for 14.8 ± 3.9 days (Group 11, n = 6). These survival times were not significant ($p>0.01$), however each group was significantly different when

compared to the control group in which pancreases were perfused with no additives (Group 7, mean survival 11.5 ± 1.7, n = 7, $p < 0.0001$).

5.5.6 The Effect of Complement on the Allograft Survival of Lymph Node Depleted Pancreases Perfused with Anti-Class II MAb's.

The rat pancreas is studded with relatively large lymph nodes which must contribute significantly to the antigenic load of the allograft upon transplantation. Furthermore, any difficulties in achieving significant immunomodulation, by attempting to deplete all the Class II positive cells within the graft (and accompanying lymph nodes), would only be potentiated by their presence. Therefore, attempts were made to surgically remove these nodes, particularly from around the splenic vein, to study the effects of prolonged ex vivo perfusion on these segmental grafts. However, because of the close proximity of the lymph nodes to the splenic vein, *complete* removal of all the nodes was difficult, time consuming and proved quite impossible. Nevertheless, a series of experiments were performed in which many of the large contaminating lymph nodes were surgically removed prior to ex vivo perfusion and transplantation, although it must be admitted that complete removal of all the contaminating lymph nodes was probably not achieved.

Six 'lymph node depleted' pancreases perfused for 3 hours with ISCR3 alone were successfully transplanted. Their allograft survival ranged from 10 to 14 days with a mean of 11.8 ± 1.6 days (Group 10, n = 6) (see Table 5.3). However, only 3 'lymph node depleted' pancreases survived the surgical manipulation and ex vivo perfusion with both ISCR3 and C'. These pancreases functioned for 12, 14 and 16 days (Group 9, mean survival 14 ± 2 days, n = 3). The difference in prolongation of allograft survival between both these groups was not significant ($p>0.03$), because the numbers in Group 9 were small. Nevertheless, there was a slight improvement in allograft survival, when C' was added to the lymph node depleted group. This contrasts to the results in 5.5.4 (shown in Fig. 5.9) where there was no difference. One could speculate that complement may play a role in the immunoalteration of perfused pancreases from

PLATE 5.4

PHOTOGRAPH OF A SECTION OF A LYMPH NODE IN A RAT PANCREAS PERFUSED WITH ANTI-CLASS II MONOCLONAL ANTIBODIES

Plate 5.4 This is a photograph of a section of a lymph node within a $LDAF_1$ pancreas perfused for 2 hours with ISCR3. Following ex vivo perfusion the section has been immunohistochemically stained for the anti-Class II MAb, using an alkaline phosphatase substrate stain. The section was also lightly counterstained with haematoxylin.

It clearly demonstrates that the anti-Class II MAb, ISCR3, labels Class II positive cells within the lymph node itself, as well as the pancreas. The Class II positive B cell germinal follicles are visible at the periphery of the node. The predominantly Class II negative T cells, occupying the centre of the node are not labelled, consequently the area appears pale.

TABLE 5.3

THE EFFECT OF ADDING COMPLEMENT TO EITHER INTACT PANCREASES PERFUSED WITH MAb OR LYMPH NODE DEPLETED PANCREATIC SEGMENTS

Group	Perfusion Period	Additive	Graft survival (days)	Mean ± SD (days)
Normal pancreatic segment				
8.	3 hours *(n = 6)*	ISCR3 and C'	12, 14, 16, 16, 17, 21	16.0 ± 3.0
11.	3 hours *(n = 6)*	ISCR3	11, 12, 14, 14, 16, 22	14.8 ± 3.9
Lymph node depleted pancreatic segments				
9.	3 hours *(n = 3)*	ISCR3 and C'	12, 14, 16	14.0 ± 2.0
10.	3 hours *(n = 6)*	ISCR3	10, 10, 12, 12, 13, 14	11.8 ± 1.6

Table 5.3 Comparison of the effect of adding rabbit complement (C') to the perfusate of $LDAF_1$ pancreases on allograft survival. Furthermore, this table shows the effect of removing the lymph nodes from pancreatic allografts prior to transplantation and the synergistic effect of adding exogenous C' to the perfusate.

There is no significant difference in allograft survival when C' is added to the perfusate containing ISCR3, following a 3 hour perfusion of intact pancreatic segments (Groups 8 and 11, $p>0.05$). However, there is a slight improvement in allograft survival when C' is added to the perfusate of lymph node depleted pancreatic segments, which was not statistically significant (Groups 9 and 10, $p>0.03$).

FIG 5.11

THE EFFECT OF ADDING COMPLEMENT ON THE SURVIVAL OF LYMPH NODE DEPLETED PANCREATIC ALLOGRAFTS PERFUSED WITH MAb

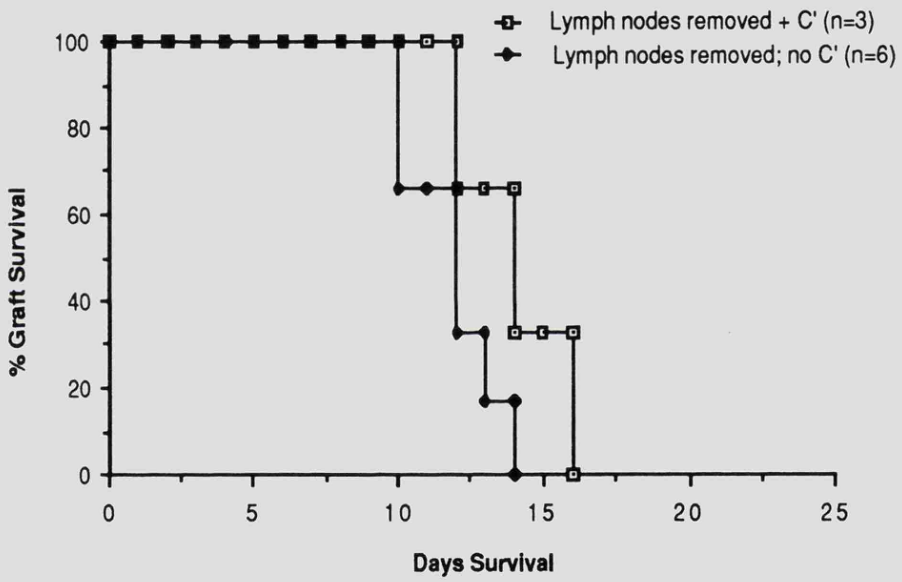

Fig. 5.11 The effect of rabbit complement (C') added to the perfusate on the survival of rat pancreas allografts depleted of their lymph nodes prior to the ex vivo perfusion.

This graph verifies that there was a slight improvement in the prolongation of allograft survival when C' was added to the perfusate of $LDAF_1$ pancreases, depleted of their lymph nodes before perfusion. This contrasts with the results shown in Fig. 5.9 which did not show any benefit upon allograft survival when C' was added to the perfusate of pancreases with their lymph nodes intact.

FIG. 5.12

THE OVERALL EFFECT OF PROLONGED PERFUSION OF PANCREATIC ALLOGRAFTS PERFUSED WITH ANTI-CLASS II MONOCLONAL ANTIBODIES

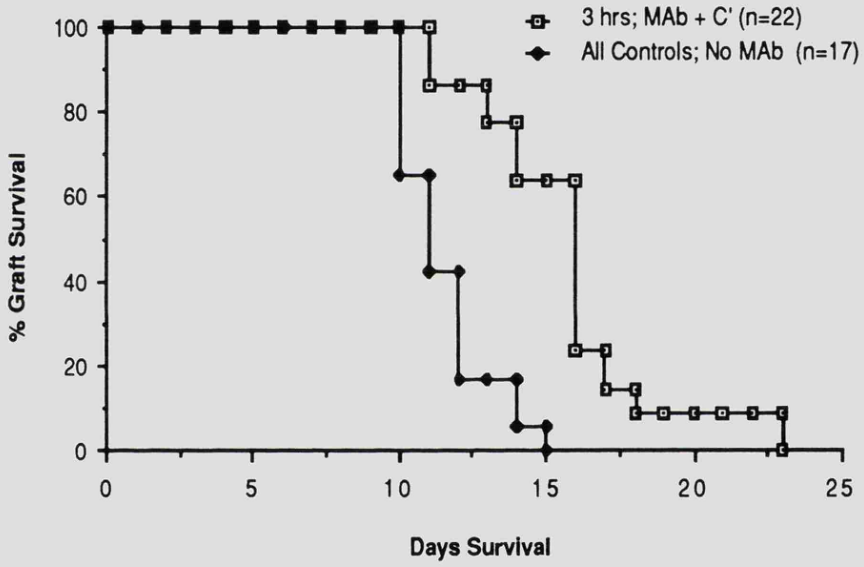

Fig. 5.12 The effect of prolonged ex vivo perfusion with anti-Class II monoclonal antibodies (MAb) and rabbit complement (C') on the survival of pancreatic allografts ($LDAF_1$ to DA).

This graph shows the results of all pancreases perfused for 3 hours with MAb, and compares their allograft survival times to all control groups combined. It clearly demonstrates that when pancreases are perfused for 3 hrs on the ex vivo circuit, with anti-Class II MAb's and C' included in the perfusate, then there is a significant prolongation of allograft survival. The immunogenicity of the allografts is reduced because of pretreatment with anti-Class II MHC antigen monoclonal antibodies.

which the lymph nodes were removed because the total numbers of Class II positive cells were significantly reduced.

5.6 SUMMARY

The optimal parameters for the ex vivo perfusion of pancreases were obtained. In this way $LDAF_1$ pancreatic segments could be perfused for three hours without incurring any significant functional damage to the grafts. All pancreases reversed the experimentally induced diabetes in syngeneic recipients for greater than 100 days. Furthermore, when the allograft survival of control pancreases perfused without MAb, with or without rabbit complement, was compared to that of untreated or unperfused allograft survival, there was no significant difference. Thus the act of perfusing the pancreases for 3 hours on the ex vivo oxygenating, normothermic circuit did not alter the immunogenicity of the glands, neither did the addition of C'.

Significant prolongation in allograft survival could be obtained, however, when $LDAF_1$ pancreases were transplanted into diabetic DA recipients following a period of ex vivo perfusion with *anti-Class II monoclonal antibodies* included in the perfusate. Indeed, perfusion for 1, 2, and 3 hours all resulted in a significant improvement in allograft survival, but the most significant difference was observed in the 3 hour perfusion groups. Prolonged perfusion with increased exposure to the MAb's was beneficial. Simple flushing of the pancreas with a combination of 14.4.4. and OX6 at 4°C, just prior to transplantation, did not result in any prolongation of allograft survival.

The addition of *combinations* of anti-Class II MAb's to the ex vivo perfusion circuit did not significantly improve the prolonged allograft survival either. Adding a 'cocktail' of anti-Class II MAb's would obviously increase the total number of antigenic binding sites and increase the likelihood of complement lysis and dendritic cell depletion. This will be discussed in more detail in the following Chapter. If lysis of the dendritic cells was not to occur, then the

probable inactivation of the Ia positive cells, by MAb labelling of the Class II MHC antigen-bearing cells within the pancreas would be increased. Suprisingly, perfusing pancreases with both 14.4.4.and OX6, together with C' for 3 hours, did not result in any longer allograft survival than in pancreases perfused with ISCR3 and C' alone, or 14.4.4. alone, for 3 hours. The specificity of the antibody used in the perfusate did not alter the allograft survival also. The prolongation of allograft survival was statistically similar when pancreases were perfused with either 14.4.4. alone or ISCR3 alone.

The effect of complement-dependent lysis, as a synergistic aid to the presumed immunoalteration by enhancing the depletion of the Ia positive cells within the perfused pancreas was variable. Indeed, when normal pancreatic segments were perfused for 3 hours with ISCR3 and C', the resulting allograft prolongation was similar to that of pancreases perfused with ISCR3 alone. However, contrasting results were obtained when pancreases, depleted of their lymph nodes prior to a 3 hour exposure to ISCR3, were perfused with complement added to the perfusate. Although the numbers were small, there was a prolongation of survival when C' was added to the perfusate of lymph node depleted pancreas grafts, though it was not statistically significant. One could speculate that the enormous quantity of Class II positive cells within the pancreas and it's accompanying lymph node population, would overwhelm any attempts at immunoalteration by the ex vivo perfusion with anti-Class II MAb's. When the majority of these Class II bearing cells were surgically removed before the perfusion procedure, then inactivating or depleting those Ia positive dendritic cells remaining actually within the interstitium of the pancreas, could result in an even better reduction in immunogenicity.

Overall, these experiments confirm that there is a significant improvement in allograft survival when $LDAF_1$ pancreases are perfused with anti-Class II monoclonal antibodies and transplanted into diabetic DA recipients. The difference is most striking when all the groups in which pancreases were perfused with MAb are compared to those pancreases perfused without MAb (see Fig. 5.12). Thus a reduction in the immunogenicity of these allografts has been achieved by ex vivo manipulation and subsequent transplantation. Other methods for determining the efficacy of the immune modulation caused by the ex vivo

perfusion with MAb, depend on *in vitro* studies of the pancreatic islets isolated from the perfused pancreases themselves. These will be discussed in the following chapters.

CHAPTER 6

IMMUNOHISTOCHEMICAL STAINING OF PANCREATIC TISSUES

6.1 INTRODUCTION

Alkaline phosphatase (AP) was amoung the enzymes which were investigated in the 1960s as potentially suitable for antibody labelling. However, AP has only been used sporadically since that time (Boorsma, 1984; Feller *et al*, 1983) with the exception of its application in the detection of epithelial membrane antigen (EMA) in carcinoma cells (Sloane *et al*, 1980) and for the characterisation of lymphocytic surface markers (Druguet and Pepys, 1977; Pepys and Pepys, 1980). In 1978, an unlabelled antibody bridge immunoalkaline phosphatase technique was reported by Mason and Sammons (Mason and Sammons, 1978) and the development of the two substrate reaction prompted the use of this technique in the following experiments. Cordell raised antibodies against AP and this enabled double labelling to be performed (Cordell *et al*, 1984). The use of other immunostaining procedures, such as ones utilising immuno-peroxidase, was limited because of the possible cross reactivity with infiltrating neutrophils and eosinophils.

Immunohistochemical analysis and the use in identifying specific antigenic proteins, carried on the surface of cells, provides yet another great application to which monoclonal antibodies can be utilised.

The principal of immunohistochemical staining depends on utilising MAb's in a bridging fashion, such that the specific antigen on the cell surface is identified and the signal amplified by bridging steps until a colour reagent, visible under the light microscope, specifically reacts with the final bridging product. This final step necessitates the use of an enzyme-substrate reaction such as AP and Fast Red, or AP and BCIP substrate, described by Leary (Leary *et al*, 1983). With this general background, an alkaline phosphatase immunohistochemical staining technique was developed and modified to study the efficacy of antibody

labelling during ex vivo perfusion.

6.2 DIRECT STAINING OF PANCREATIC TISSUES AND ISLETS

The pancreases or pancreatic islets to be studied were prepared carefully, with fat and contaminating tissues removed, and placed in the correct orientation in small plastic specimen containers. Tissue mounting medium, O.C.T. (Gibco Laboratories, Grand Island, New York), was placed around the specimens which were subsequently snap frozen in liquid nitrogen (-196°C) for five minutes. Cryostat sections 3-6μm thick were cut on a microtome and mounted on albuminised glass slides to prevent dissociation of the sections from the slides. (The glass slides were dipped in water containing 0.05% albumin, Sigma Chemicals, and air dried overnight in an oven). The cryostat sections were then fixed in acetone for fifteen minutes and subsequently washed in phosphate buffered saline (PBS, Gibco), pH 7.6, for ten minutes. The tissue sections were incubated with the primary MAb's, diluted in PBS, with 1% fetal calf serum (FCS), and 0.1% sodium azide to a final concentration of 1:1000, for one hour at room temperature. Following a second ten minute wash in PBS, the tissue sections were incubated with a rabbit anti-mouse monoclonal antibody conjugated with alkaline phosphatase (RAM-AP, Dako Laboratories, Burlingame, CA.) for thirty minutes. Occasionally, cross reactivity with the secondary MAb was blocked by an incubation with rabbit serum, diluted 1:10, for 30 minutes, before this secondary step. Following these monoclonal incubations the sections were washed with PBS for ten minutes and then developed with an alkaline phosphate substrate stain (Vector Kit 11, Vector Laboratories, Burlingame, CA.) which stained positive cells dark brown or black in ten to twenty minutes. Cross reactivity with endogenous alkaline phosphatase was inhibited with the addition of 0.1M Levamasole (Sigma L-9756) to the final substrate stain mix, as described by Ponder *et al*, in 1981. The staining reaction was stopped by washing and the sections counterstained with haematoxylin. Finally, the sections were fixed with Immunomount (Sigma) and covered with glass slides.

PLATE 6.1

PHOTOGRAPH OF A SECTION OF PANCREAS LABELLED WITH AN ANTI-CLASS II MAb.

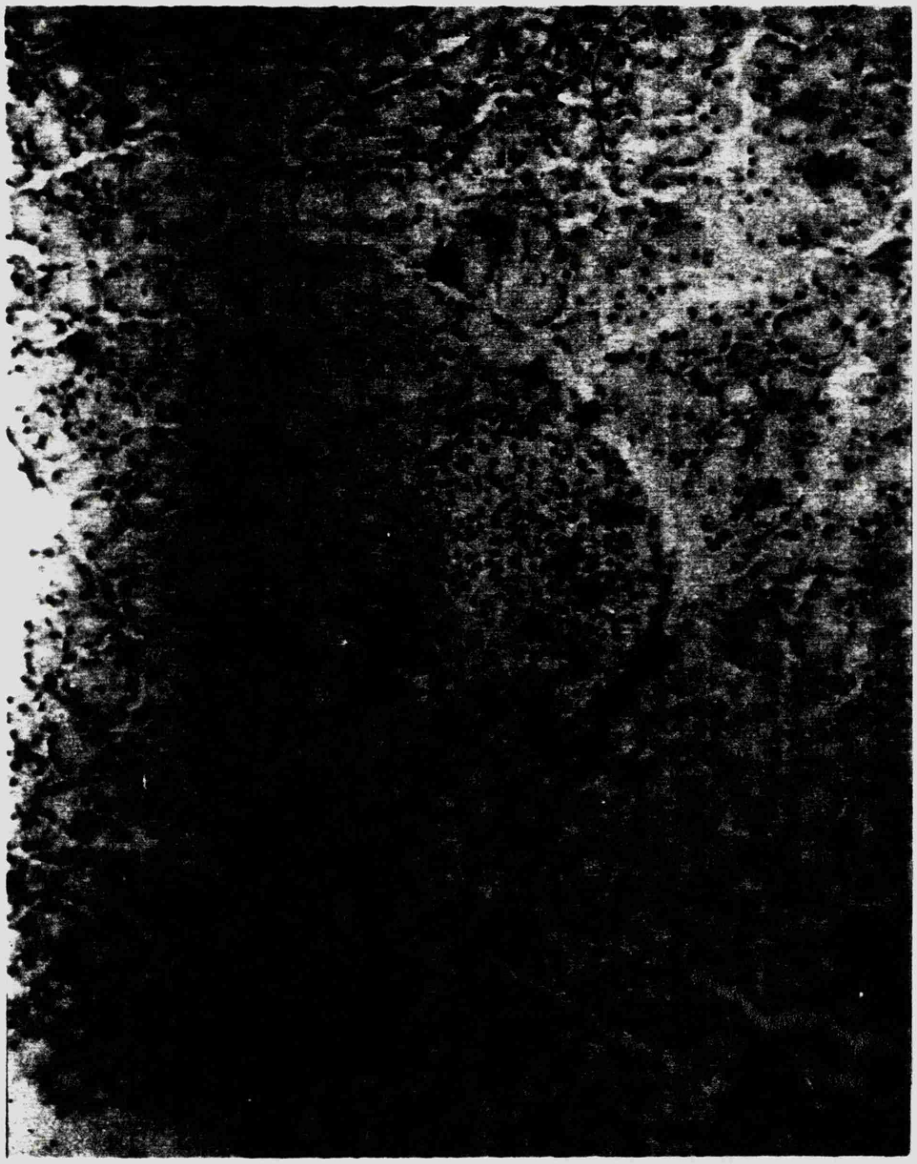

Plate 6.1 This is a photograph of a section of $LDAF_1$ rat pancreas immunohistochemically labelled with an anti-Class II monoclonal antibody, OX6, stained black using an alkaline phosphatase reagent. The section was counterstained with haematoxylin.

Cells with dendritic morphology are clearly identified in and around the pancreatic islet (centre), but also throughout the interstitium of the pancreas.

PLATE 6.2

PHOTOGRAPH OF A SECTION OF A RAT PANCREATIC ISLET DEMONSTRATING CLASS II POSITIVE CELLS

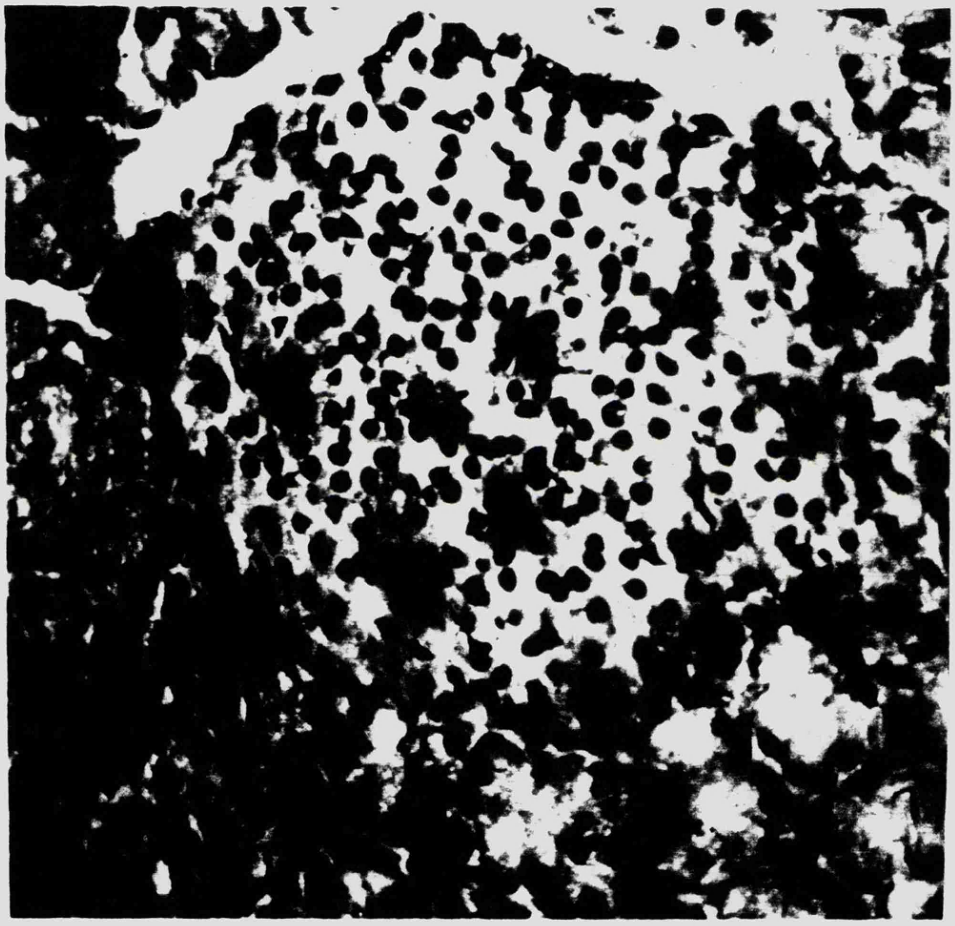

Plate 6.2 This is a photograph of a section of $LDAF_1$ rat pancreatic islet, immunohistochemically labelled with an anti-Class II monoclonal antibody, 14.4.4., stained black using an alkaline phosphatase reagent. The section was lightly counterstained with haematoxylin.

Cells with dendritic morphology were identified within the islet. On this cross section there are about 10 Class II positive cells shown within the islet.

6.3 DOUBLE LABELLING OF PANCREATIC TISSUE SECTIONS

To identify two or more different cellular subtypes expressing different antigenic determinants on the tissue sections, a sequential or 'double labelling' staining technique was developed. The frozen section preparations were incubated first with the primary and subsequently with the secondary monoclonals as described above, and developed with the brown/black substrate stain, Vector II. However, before counterstaining with haematoxylin, a second labelling procedure was employed using a different primary MAb and a different substrate stain. The tissue sections were thoroughly washed in PBS for fifteen minutes following development with the brown/black stain but then incubated with a second and different primary MAb for one hour. After washing, the same secondary MAb (RAM-AP, 1:30 dilution) was employed for thirty minutes, but this time followed by a different alkaline substrate stain which utilized Fast Red as the colour indicator (Sigma, Cat. No. F-1500). For the final Fast Red stain, 2mg of Naphthol AS-MX Phosphate (Sigma, Cat. No. N-5000) was dissolved in 0.2 mls of dimethyl formamide and diluted in 0.8 mls of Tris HCl buffer, pH 8.2. Just prior to staining 1 mg of Fast Red was dissolved in this solution and filtered onto the tissue sections. The slides were incubated for twenty minutes and finally washed in PBS. In this way, the cells bearing the epitope which was recognized by this second primary MAb stained *red* only.

However, if the cells expressed the antigenic determinants of both the primary antibodies used, then these cells would theoretically stain both red and black. Actually, because of the density of the brown/black stain, the cells stained either brown/black alone or brown/black with a red halo. Once again, these slides were counterstained with haematoxylin and mounted with a glass slide. Eosin was not used as the red colour would interfere with the fast red stain (see Fig. 6.4a, 6.4b, and 6.4c).

6.4 LABELLING OF CLASS II POSITIVE CELLS DURING EX VIVO PERFUSION

Following a period of ex-vivo perfusion, with or without MAb, the pancreases were flushed with 1 ml of ice cold saline and snap frozen in liquid nitrogen. Cryostat sections were taken and incubated directly with rabbit anti-mouse (RAM-AP) for thirty minutes. The primary step at this stage was therefore been omitted but in this way the secondary RAM-AP would label the primary MAb already on the Class II cells in the pancreas. These sections were washed in PBS and subsequently developed with the brown/black alkaline phosphate substrate stain. To assess whether all the Class II antigen positive cells were infact labelled with MAb during the period of ex-vivo organ perfusion, sections of pancreases perfused with one MAb were developed with a black stain, and then these *same sections reincubated* with a second anti-Class II MAb. They were subsequently developed with the second red staining alkaline phosphate substrate stain.

This double labelling sequential staining technique determined the efficacy of penetrance of the MAb into the intact organ during a specific perfusion period, as the cells labelled during the period of ex-vivo perfusion would stain black, and any additional cells not labelled during the perfusion period would be stained red. Cells labelled during both the perfusion and with the subsequent incubation would stain both black and red.

6.5 COUNTING OF CLASS II MHC ANTIGEN POSITIVE CELLS

Following immunohistochemical staining and final mounting with the glass cover slips, the numbers of positively labelled cells were counted using a grid micrometer (Olympus, Cat. No. 4067), placed in one of the eye pieces of the light microscope. Areas on the tissue sections were randomly chosen and the average number of cells from at least twelve different areas, from at least three different slides was taken as an indication of the number of positively stained cells

TABLE 6.1

COMPARISON OF THE NUMBERS OF CLASS II POSITIVE CELLS IN DIFFERENT RAT STRAINS

Rat Strain	Monoclonal Antibodies		
	14.4.4.	OX6	ISCR3
LEW	62 ± 8*	65 ± 9	63 ± 12
$LDAF_1$	72 ± 16	76 ± 21	61 ± 12
DA	68 ± 24	64 ± 18	71 ± 25
$LBNF_1$	64 ± 8	66 ± 18	62 ± 11

* Number of labelled cells per square millimeter ± standard deviation.

Table 6.1 Immunohistochemical labelling of cells in the rat pancreas with anti-Class II monoclonal antibodies. This table shows the comparison of the number of Class II antigen bearing cells in the pancreases of different rat strains as determined by three different monoconal antibodies, 14.4.4., ISCR3, and OX6. The results confirm that there are equal numbers of Class II positively stained cells (dendritic cells) in each pancreas, as the numbers of positive cells did not vary significantly in any group ($p<0.001$).

TABLE 6.2

LABELLING OF CLASS II POSITIVE CELLS DURING PERFUSION

Perfusion period	Concentration of MAb in perfusate	No. of cells labelled during perfusion	Additional Cells labelled on tissue sections
30 mins	1 μg/ml	30 ± 3	35 ± 6
1 hour	1 μg/ml	55 ± 4	10 ± 3
1 hours	20 μg/ml	61 ± 4	3 ± 2
3 hours	20 μg/ml	61 ± 4	0 ± 2

Table 6.2 Comparison of the numbers of Class II positive cells per square millimeter in LEW rat pancreases labelled during different perfusion periods with varying concentrations of the anti-Class II MAb 14.4.4. This table shows that maximal labelling of Class II antigen bearing cells occurs when the MAb has a concentration of 20 μg/ml and the pancreas is perfused for three hours although the numbers of cells labelled at one hour were not significantly different ($p>0.5$).

FIG. 6.1

LABELLING OF CLASS II POSITIVE CELLS DURING EX VIVO PERFUSION

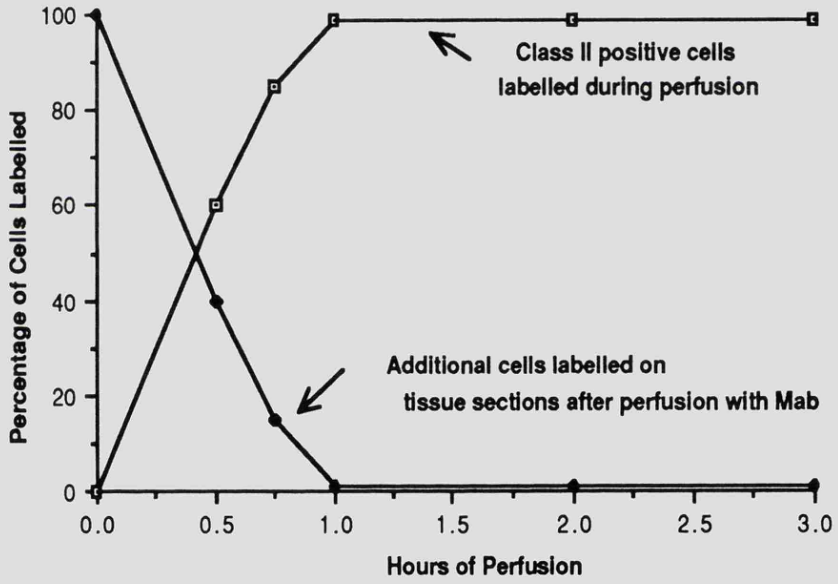

Fig. 6.1 Graph showing the percentage of Class II positive cells within a rat pancreas labelled during ex vivo perfusion. At 30 minutes only 60 % of the cells were labelled with OX6 (see text), as determined by alkaline phosphatase immunohistochem -ical labelling. At one hour virtually all the cells were labelled with MAb, and no additional cells could be identified with a second MAb probe on those tissue sections.

in that particular treatment group. In this way, the numbers of cells labelled with any MAb could be assessed and expressed as cells/mm^2.

6.6 DONOR PRETREATMENT WITH CYCLOPHOSPHAMIDE AND IRRADIATION

To establish whether in this strain combination other previously reported methods for reducing dendritic cell content of allografts were employed in one study. McKenzie and colleagues reported successful allograft prolongation when donor animals were pretreated with a combination of cyclophosphamide and gamma irradiation (McKenzie *et al,* 1984). In an attempt to reproduce this effect, donor animals were pretreated with cyclophosphamide, 200 mg/kg intraperitoneally, five days before donor pancreatectomy. In addition, the same animals were given 1000 rads of whole body gamma irradiation three days prior to pancreatectomy. Whole body irradiation had been proven to reduce the numbers of Class II positive interstitial dendritic cells within kidney allografts (McKenzie *et al,* 1984).

The pancreatic grafts were transplanted from pretreated LDAF$_1$ donors into non-immunosuppressed diabetic DA hosts. The allograft survival was determined as before with daily measurements of blood glucose levels. Furthermore, other pancreases were removed on days 2, 3, and 4 following this combined pretreatment and analysed for their dendritic cell content.

6.7 RESULTS

6.7.1 Direct staining of pancreatic tissues

Cells with dendritic cell morphology were identified in the pancreas by immunohistochemical labelling of fixed pancreatic tissue sections using anti-Class II monoclonal antibodies. The Ia positive cells seemed to concentrate around blood vessels but were also distributed widely throughout the acinar tissue and

PLATE 6.3

PHOTOGRAPH DEMONSTRATING LABELLING OF CLASS II DENDRITIC CELLS DURING PERFUSION WITH ANTI-CLASS II MAb

Fig. 6.3 Photomicrograph of a section of $LDAF_1$ rat pancreas perfused with the monoclonal antibody (MAb) 14.4.4. for 2 hours. The section was incubated with rabbit anti-mouse MAb, developed with a black alkaline phosphatase substrate stain and counterstained with haematoxylin.

It can be seen that Class II positive cells were clearly labelled throughout the pancreas (and islets, not shown here) during the perfusion period with the anti-Class II MAb.

PLATE 6.4 (a)

PHOTOGRAPH DEMONSTRATING DOUBLE LABELLING OF CLASS II POSITIVE CELLS WITHIN A RAT PANCREAS

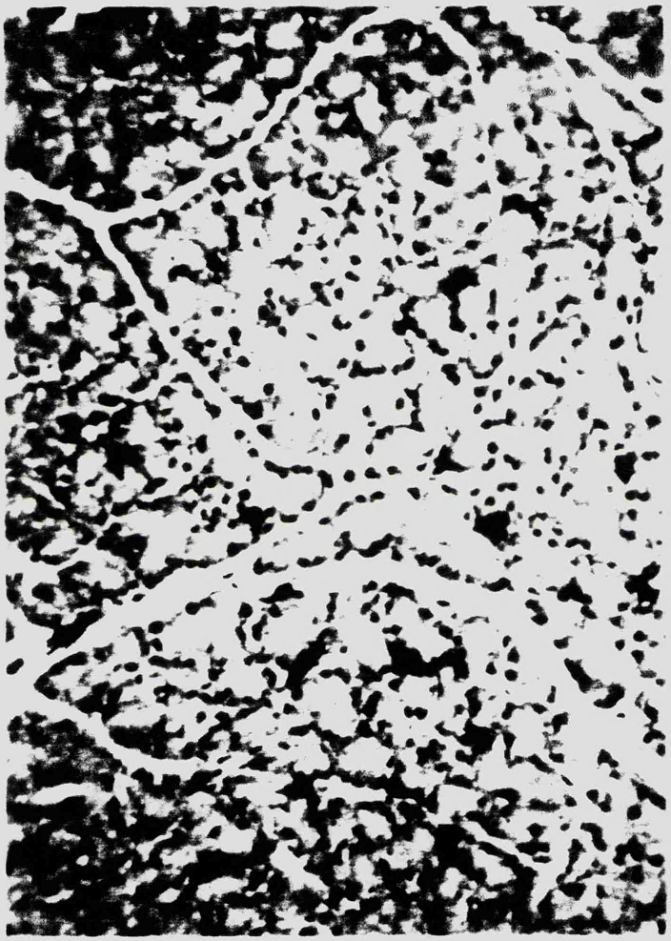

Fig. 6.4 (a) Photomicrograph of a section of a $LDAF_1$ rat pancreas demonstrating the double labelling of the Class II positive cells within the pancreas. The pancreas was perfused with 14.4.4. for three hours, to label the dendritic cells, and developed with a black alkaline phosphatase substrate stain. Subsequently, the tissue section was incubated with OX6, developed with a red stain, and lightly countertained with haematoxylin.

It can clearly be seen that all the Class II positive cells were labelled both during the perfusion period and subsequently by incubation with the second MAb. This is evident because the cells identified were stained both black and red.

PLATE 6.4 (b)

PHOTOGRAPH DEMONSTRATING DOUBLE LABELLING OF CLASS II POSITIVE CELLS WITHIN A RAT PANCREAS

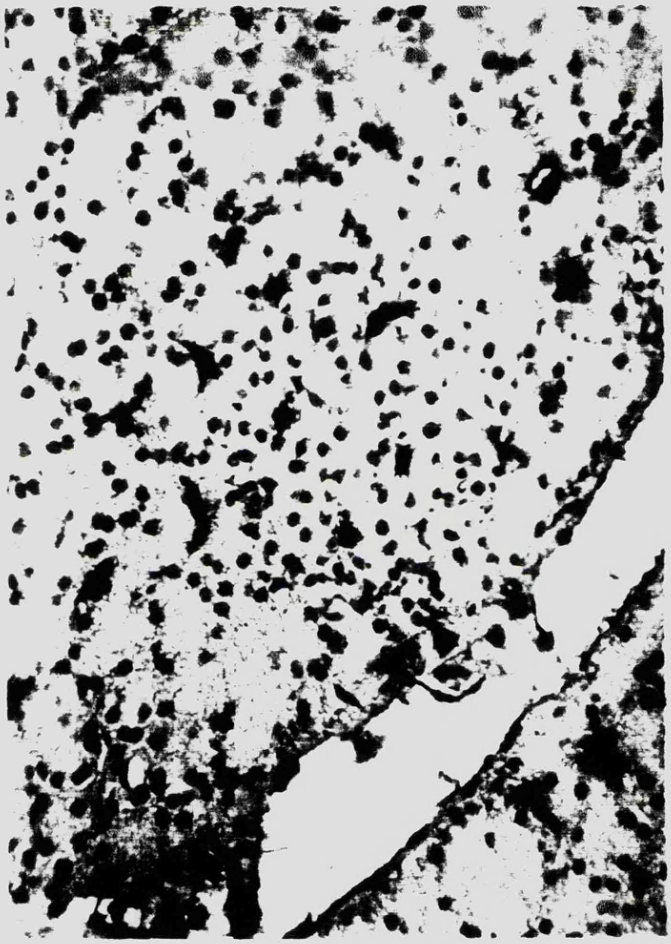

Fig. 6.4 (b) Photomicrograph of a section of a $LDAF_1$ rat pancreas demonstrating the double labelling of the Class II positive cells within the pancreas. The pancreas was perfused with ISCR3 to label the dendritic cells, and developed with a black alkaline phosphatase substrate stain. Subsequently, the tissue section was incubated with OX6, developed with a red stain, and lightly countertained with haematoxylin.

The section demonstrates that the Class II positive cells within the pancreas and islet (centre) were labelled during perfusion and also by subsequent incubation with the second MAb. The cells identified stained both black and red.

Note that the vascular endothelium, lining the vein, was not labelled by either MAb, verifying it did not express Class II MHC antigens.

PLATE 6.4 (c)

PHOTOGRAPH DEMONSTRATING DOUBLE LABELLING OF CLASS II POSITIVE CELLS WITHIN A RAT PANCREAS

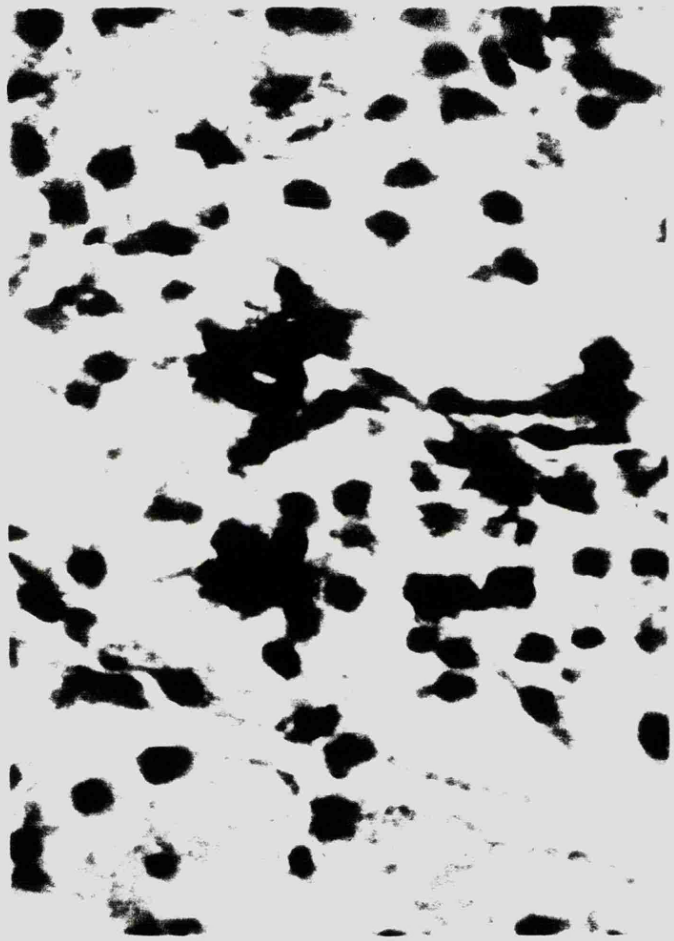

Fig. 6.4 (c) Photomicrograph of a section of a $LDAF_1$ rat pancreas demonstrating the double labelling of the Class II positive cells within the pancreas. The pancreas was perfused with 14.4.4. to label the dendritic cells, and developed with a black alkaline phosphatase substrate stain. Subsequently, the tissue section was incubated with OX6, developed with a red stain, and lightly countertained with haematoxylin.

This high power view demonstrates that the Class II positive cells within the pancreas were labelled during perfusion and also by subsequent incubation with the second MAb. The cells identified black with a red halo.

PLATE 6.5

PHOTOGRAPH DEMONSTRATING PANCREATIC DUCTAL EPITHELIUM DOES NOT EXPRESS CLASS II MHC ANTIGENS

Fig. 6.5 Photomicrograph of a section of a $LDAF_1$ rat pancreas demonstrating that the ductal epithelium in the rat pancreas does not express Class II MHC antigens. This section was incubated with 14.4.4. and subsequently with OX6. Despite labelling of some Class II positive interstitial cells, the pancreatic ductal cells did not label with either MAb.

could clearly be identified in pancreatic islets (Plate 6.1 and 6.2).

The numbers of Ia +ve cells per square millimeter labelled with 14.4.4., OX6, and ISCR3 are shown in Table 6.1. Although the numbers of stained cells varied moderately in different areas of each section, the number of cells reactive with anti-Class II MAb did not vary significantly between animals or strains. In addition, each monoclonal antibody, although specific for a different antigen epitope, labelled the same number of cells in the pancreatic tissue sections.

6.7.2 Addition of MAb to the Perfusate during ex vivo perfusion

Rat pancreases were next exposed to the anti-Class II monoclonal antibodies during perfusion of the viable organ rather than after tissue section fixation and preparation. An equal number of cells were labelled whether by exposure to a MAb during a three hour perfusion with MAb at a concentration of 20 μg/ml in the perfusate or by incubation of fixed tissue sections with the monoclonal antiboby, as indicated by the Table 6.1. Table 6.2 shows that at low (1 μg/ml) concentrations of antibody, a thirty minute or even a one hour perfusion was not sufficient to label all the Class II antigen positive cells as more cells could be labelled by additional immunohistochemical staining of MAb, using the double labelling technique described in the methods section. Furthermore, MAbs were clearly in excess at concentrations of 20 μg/ml, even when the pancreas was perfused for up to three hours. After a three hour perfusion of a pancreas with 14.4.4. the "spent" perfusate could be used to label denritic cells in a second pancreas without a decrease in staining intenstity, and could lyse rat splenic B cells in a complement dependent cytotoxic assay to a titre of greater than 1 : 250. To further determine the efficacy of the ex vivo perfusion, double labelling experiments were performed where one of the anti-Class II MAb's was included in the perfusate during the ex-vivo perfusion period and tissue sections prepared and stained by the alkaline phosphatase technique described. The tissue sections were then re-exposed to the same MAb or different anti-Class II MAb'sand restained with a second substrate stain of contrasting colour.

FIG. 6.2

SURVIVAL OF PANCREATIC ALLOGRAFTS FROM DONORS PRETREATED WITH CYCLOPHOSPHAMIDE AND DXT

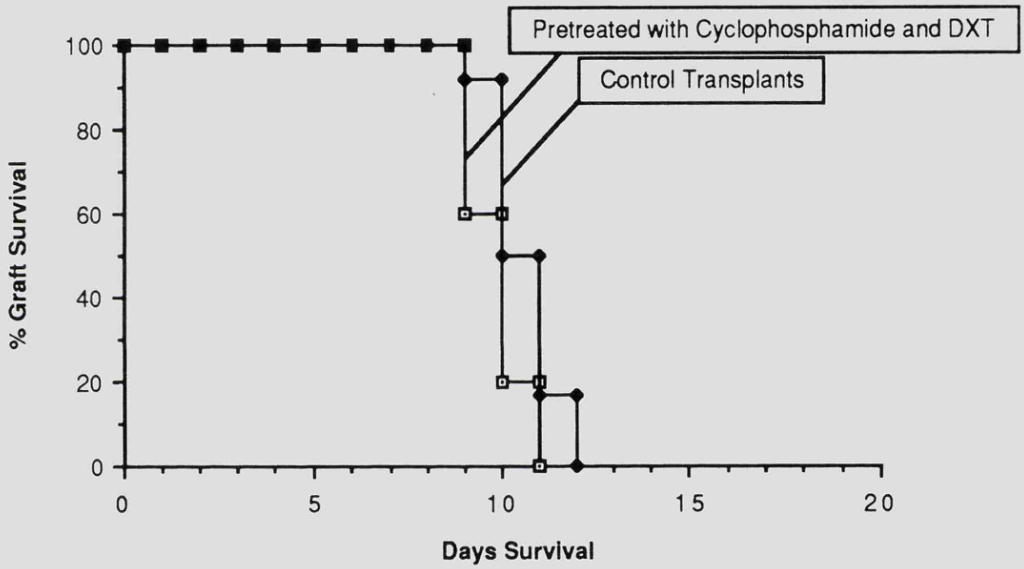

Fig. 6.2 Graph comparing the survival of pancreatic allografts removed from $LDAF_1$ donors pretreated with cyclophosphamide (200 mg/kg intra peritoneally, day -5) and irradiation (DXT, 1000 rads, given day -3) and transplanted into diabetic DA recipients compared to untreated controls.

It can be seen that there was no significant improvement in allograft survival when pancreases were removed from pretreated donors (mean survival 9.8 ± 1.0 days, n=5) compared to untreated controls (mean survival 10.9 ± 0.9 days, n=12)(p>0.5).

FIG. 6.3

EFFECT OF CYCLOPHOSPHAMIDE AND IRRADIATION PRETREATMENT ON NUMBERS OF CLASS II POSITIVE CELLS

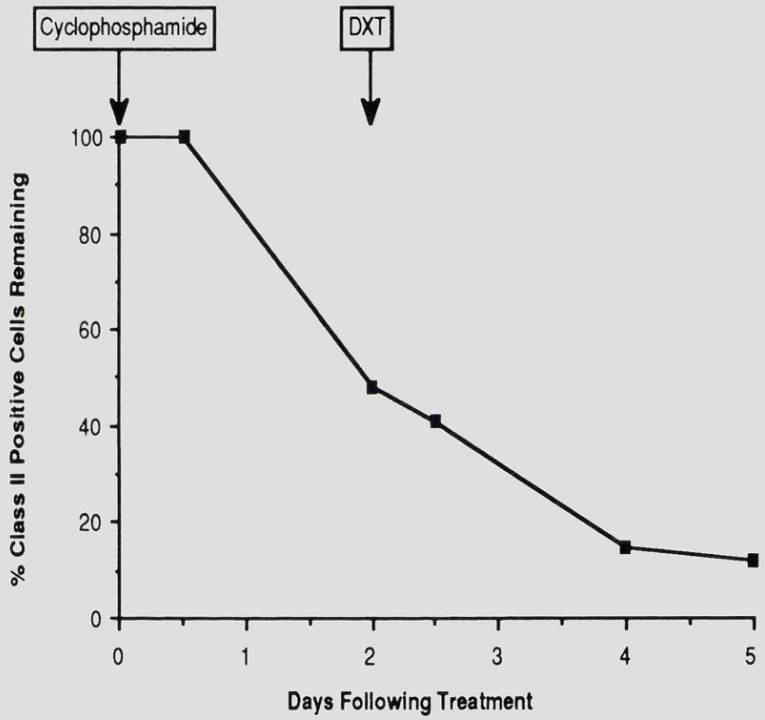

Fig. 6.3 Graph demonstrating the effect on the Class II positive cell content of pancreases removed from $LDAF_1$ animals pretreated with cyclophosphamide (200 mg/kg, day -5) and donor irradiation (DXT, 1000 rads, day -3).

It can be seen that there is a significant reduction in the numbers of Class II cells following the combined pretreatment of donors. However, even at day 5 there are 12-15% of Class II positive cells remaining in the pancreas.

After a period of 1 or 3 hours of ex vivo perfusion (20 μg MAb), only 0 - 2 additional MAb reactive cells per mm^2 could be detected following the second labelling, which were not labelled during the period of ex-vivo perfusion of the viable organ, indicating virtually complete access of the MAb's during perfusion to Class II antigen expressing cells in the pancreas. There was no difference in the small number of additional cells which could be detected by the second MAb whether this was an I-A or I-E reactive MAb.

6.7.3 Effect of donor pretreatment with cyclophosphamide and irradiation on the survival of pancreatic allografts and dendritic cell content of the pancreas.

For the transplant study, eight animals were pretreated with cyclophosphamide and irradiation. Three animals died as a result before pancreatic transplantation could be performed. Of the five successful allotransplants, all functioned post operatively for a period of time. Grafts were rejected, as determined by a rise in blood sugar over 200 mg/dl, on 9, 9, 10, 10, and 11 days respectively (mean survival 9.8 ± 1 days, n=5). This was not statistically different from the survival times of untreated contol transplants (mean survival 10.9 ± 0.9 days, n=12). The results are shown in Fig. 6.2.

Furthermore, ten animals were pretreated in a similar manner and subsequently two animals each day thereafter were sacrificed and the pancreases studied immunohistochemically for the presence of Class II positive cells. In this way the effect of this pretreatment regimen on the dendritic cell content within the pancreas could be serially determined. The grafts were slightly injected and the spleens were grossly shrunken as a result.

Fig. 6.3 graphically portrays the significant reduction in the numbers of positively stained cells within the pancreases pretreated with both cyclophosphamide and irradiation. However, even five days following cyclophosphamide pretreatment and three days following irradiation, there were significant numbers of Class II positive cells detectable by anti-Class II probes within the pancreases. Indeed, 12-15% of the original numbers counted were present.

6.7 DISCUSSION

The immunohistochemical techniques employed, demonstrated Class II MHC positive cells within the interstitium and the islets of all the species of rat studied. They were of 'dendritic' morphology and similar in appearance to the dendritic cells referred to in Chapter 3, described by Steinman (Steinman *et al,* 1980).

Using the MAb's, 14.4.4., ISCR3, and OX6, the numbers of positively labelled cells were not significantly different in pancreases removed from $LDAF_1$, LEW, LBN or DA rats. Since each specificity of 14.4.4. and ISCR3 is different from that of OX6 (see Chapter 4.6), it can be concluded that in the animal sections studied, since the numbers of positively stained cells were similar with each different MAb used, the Class II positive cells shared common determinants. This is important because if the immunoalteration of the pancreas allografts is dependent on the total inactivation of all the Ia bearing cells within the gland, then all populations of these Ia bearing cells should be lysed or inactivated; there should be no subpopulation of Class II dendritic cells left unlabelled.

The double labelling experiments also confirm this finding. Since only 0 - 2% of additional cells could be identified with a second MAb in tissue sections labelled with another, then each primary MAb used labelled virtually all the Class II cells within the pancreas.

Of paramount importance are the results of the labelling during ex vivo perfusion. The immunological effect of ex vivo perfusion is totally dependent on the inactivation (or removal) of *all* the Class II positive cells within the allograft. In fact, it is this reason why the double labelling experiments were established. Even though the numbers of Ia positive cells counted on tissue sections of pancreases perfused with an anti-Class II MAb, were statistically similar to those counted on unperfused specimens, the efficacy of the perfusion apparatus had to confirmed. Indeed, it was. No additional Class II positive cells could be determined when tissue sections of pancreases exposed to one MAb during perfusion were subsequently exposed to another.

All the Class II positive cells detectable within the pancreas were labelled during a 1, 2 or 3 hour period of ex vivo perfusion with antibody.

Furthermore, even when rabbit complement was added to the perfusate, the total numbers of Class II positive cells remained constant, despite *in vitro* evidence that 14.4.4. and ISCR3 fixed C' and lysed virtually 100% of rat splenic B cells, which are Class II MHC antigen positive. [The *in vitro* studies for complement lysis were performed in the laboratory by Marguerite Buckingham, data not shown.] There was no reason to assume that similar destruction of Ia positive cells could not occur during prolonged exposure to anti-Class II MAb's on the ex vivo circuit. The blood was oxygenated and stringently kept at normothermic temperatures for optimal complement lysis. Because white cell-depleted blood was used, which was also diluted to a haematocrit of 20%, accurate assessment of the lysis of circulating Class II bearing cells remaining in the blood/perfusate, was difficult. It was similarly frustrating to assess whether the Class II positive cells within the pancreas were simply labelled or actually lysed.

Following a 3 hour perfusion period, 1 ml of trypan blue was added to the perfusate with the knowledge that the killed cells should not exclude the dye and therefore appear deep blue on subsequent microscopy. Viable cells, including the pancreatic parenchymal cells, would exclude the dye and appear almost clear. This study proved to be futile. Not only was the attempt at finding a single dendritic cell analogous to the needle in the haystack, but occasionally the trypan blue precipitated micro-crystals which confused the haystack further.

The actual presence of the MAb on the cell surface also inhibited the accurate assessment of the viablity of these Ia positive cells. The mere fact that these cells were labelled with an intense black stain occluded accurate assessment of nuclear staining by high powered microscopic examination. Nevertheless, because the results from the perfusion and transplant experiments confirm allograft prolongation, the immunomodulation was at least dependent on the Class II antigens of the graft being blocked by the MAb used in the perfusate. Whether subsequent lysis of the cells occured following transplantation is yet to be determined.

Finally, the results of the cyclophosphamide and irradiation pretreatment experiments are interesting. No prolongation of allograft survival was achieved

by this pretreatment regimen, but this result was not altogether suprising. The immunohistochemical analysis of the pancreases revealed that there were still numerous Class II positive cells present at the time of allografting. It is this finding that strengthens the arguement that the ex vivo perfusion of the glands with anti-Class II MAb inactivates or kills the dendritic cells, since this particular technique of pretreatment does result in allograft prolongation.

CHAPTER 7

MIXED LYMPHOCYTE CULTURES (MLC) AND MIXED ISLET LYMPHOCYTE CULTURES (MILC)

7.1 INTRODUCTION

It is now widely accepted that T lymphocytes do not respond directly to a variety of soluble and insoluble antigens without the presence of Ia-bearing accessory cells or antigen-presenting cells (APC) (Unanue, 1981). Rather less is known about the role of the APC in the induction of responses to histocompatibility antigens. Stuart *et al*, and Lechler and Batchelor, have shown that the immunogenicity of allogeneic rat kidneys is substantially reduced in some strain combinations by the loss of donor strain dendritic cells (Stuart *et al*, 1971; Lechler and Batchelor, 1982). Enhanced allogeneic kidneys retransplanted to normal host strain rats were accepted more readily if they had been in residence in their immunosuppressed primary hosts for at least 4 weeks. Immunogenicity could be restored by the administration of small numbers of donor strain dendritic cells. Lechler and Batchelor postulated that sensitisation to alloantigens may be accomplished in two ways: (a) by direct presentation by donor APCs, and (b) by the aquisition and representation of alloantigens by host APCs, the former likely to be the dominant pathway in normal responses.

The only direct evidence for the presentation of alloantigens by host cells stems from *in vitro* studies. Weinberger demonstrated induction of secondary cytotoxic T lymphocytes by allogeneic cell membranes or lyposomes containing Class I molecules, in the absence of donor APCs (Weinberger, 1984). Golding and Singer showed that either responder or stimulator APCs facilitated the development of alloreactive cytotoxic T cells, and concluded that the responder APCs were apparently dependent on antigen processing for they, but not donor APCs, were inhibited by treatment with chloroquine (Golding and Singer, 1984). This was also corroborated by others (Finnegan *et al*, 1984). Further, responder APCs did not present antigens on paraformaldehyde-fixed

stimulator cells, indicating that shedding of antigens from the stimulator cell surface is a critical factor. Sherwood *et al* analysed the role of alloantigen presentation by hosts cels *in vivo* using a skin allograft and cell transfer model. Their results show that host APCs take up and present donor major histocompatibility (MHC) antigens early in the induction of the response to skin allografts.

The following experiments were designed to discover whether or not APCs were necessary and solely responsible for the initiation of alloaggression, and to assess the effect of removing or disabling such Ia bearing cells on the stimulation of allogeneic T cells in mixed lymphocyte cultures between Ia disparate lymphoid cell populations.

Furthermore, the capacity of islets to stimulate allogeneic T cells in mixed islet lymphocyte cultures will be determined. The cells within the pancreatic islets responsible for the initiation of the allogeneic proliferative response are the APCs resident within the islets. It will be shown that rat pancreatic islets contain between 10 and 50 Class II antigen positive cells, and that using 100 large, hand-picked islets provides the equivalent stimulation of 4 x 10^3 splenic adherent cells, and enough to stimulate an *in vitro* allogeneic proliferative response.

7.2 PREPARATION OF SPLENIC T CELLS

7.2.1 Outline

Pure T cells were obtained from a sterile spleen. Initially, a single cell suspension of spleen cells were passaged over iron particles which removed macrophages by adherence. Other Class II antigen bearing cells, including Class II positive macrophages, were removed by subsequent incubation with an anti-Class II MAb and rabbit complement (C'). The resulting cell suspension was totally devoid of Class II positive B cells, macrophages and dendritic cells and thus an almost pure suspension of T cells was obtained.

7.2.2 Method

A population of T cells were prepared from the spleens of either $LDAF_1$ or DA rats. Under sterile conditions, a spleen was removed from the animal, and placed in 10 mls of Dubecco's Modified Essential Media (DMEM, Gibco) supplemented with 10% fetal calf serum (FCS), and ground to a single cell suspension using a sterile glass tissue grinder. The cellular suspension was carefully layered over 4 mls of Histopaque (specific gravity 1.077, Gibco) and centrifuged at 1200 G for 20 minutes. Lymphocytes and contaminating macrophages settled at the interface while other leucocytes together with the erythrocytes, were spun through the density gradient and formed a pellet at the bottom of the tube. The cells at the interface were aspirated, washed in DMEM with 10% FCS, transferred to a 50 ml Falcon tube (Becton Dickinson Labware, N.J.) and brought to a final volume of 40 mls.

Ten mls of this cellular suspension were transferred to each of four 15 ml test tubes containing 200 mg of Carbonyl Iron (Sigma, St. Louis, MO.). [Note: These clean glass tubes were carefully prepared in advance by placing the iron particles at the bottom of the tube and filling the tubes with nitrogen gas for at least one minute. If this step was omitted the iron particles tended to clump together and optimal adherence could not be accomplished].

The 4 tubes containing suspensions of lymphocytes were thoroughly mixed with the iron particles and immediately transferred to a 20 ml Petri dish (Becton Dickinson) taking care to completely cover the bottom of the dish with the mixed suspension. This allowed for optimal adherence of macrophages, B lymphocytes and dendritic cells to the carbonyl iron and the sides of the dish. The petri dishes were incubated for one hour at 37°C, allowing optimal time for the phagocytosis of the iron particles by the macrophages. The carbonyl iron particles were subsequently drawn to the side of the dish using a powerful magnet. The media was aspirated into another Falcon tube and the iron filings washed thoroughly. The washings together with the aspirated media, containing the non adherent T cells, were saved. This cellular suspension now consisted mainly of T cells, but the population was probably contaminated with B cells and other Class II positive cells including dendritic cells and macrophages which were either non adherent or had become so following the incubation period.

FIG. 7.1

MIXED LYMPHOCYTE CULTURE DEMONSTRATING ALLOGENEIC PRESENTATION OF CLASS II MHC ANTIGEN

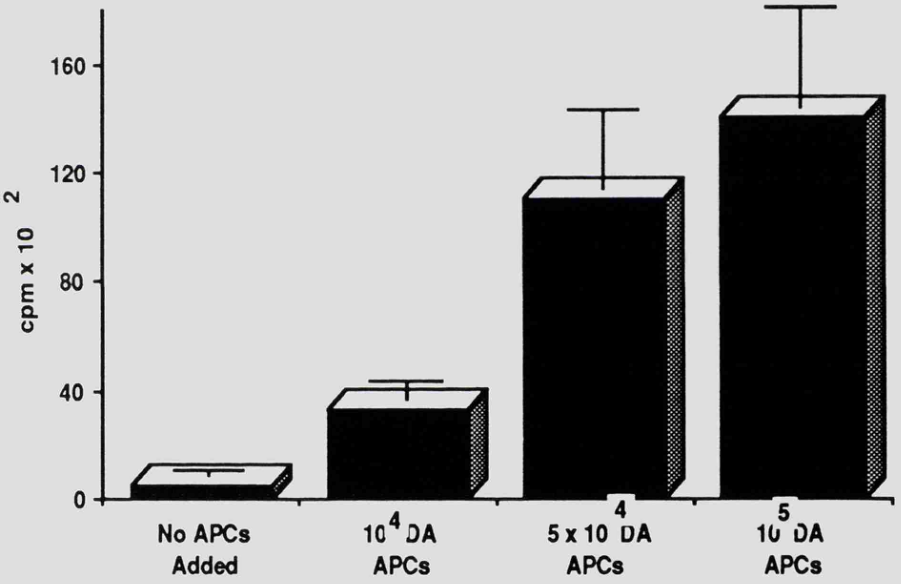

Fig 7.1 MLC between 10^5 irradiated stimulator $LDAF_1$ T cells and 10^5 responder DA T cells. Increasing numbers of responder-strain antigen presenting cells (APCs) were added to each combination of T cells.

It can be seen that when no APCs were added to the *in vitro* cultures, there was no proliferation of DA T cells. However, with increasing numbers of DA APC's added to each experiment the stimulation and proliferation of responder strain T cells, as measured by tritiated thymidine uptake, was dependent on the numbers of APCs added. The increase in thymidine uptake in each experiment was significantly different from that in any other experiment ($p<0.001$).

FIG. 7.2

MIXED LYMPHOCYTE CULTURE COMPARING THE EFFICACY OF ANTIGEN PRESENTATION BETWEEN SYNGENEIC AND ALLOGENEIC ANTIGEN PRESENTING CELLS

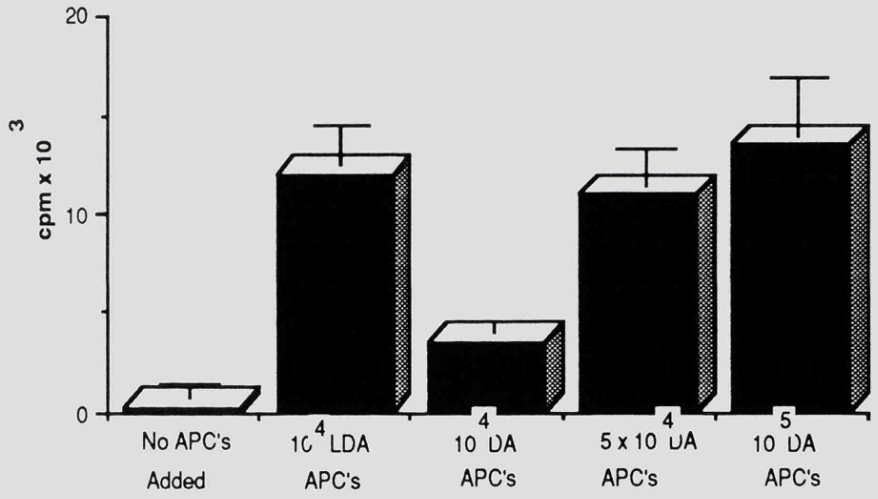

Fig 7.2 MLC between 10^5 $LDAF_1$ T cells and 10^5 DA responder T cells. This graph shows the comparison of the stimulatory capacity of stimulator-strain LDA accessory cells (APC) to that of DA responder-strain APCs.

The results demonstrate that presentation of Class II MHC antigen by syngeneic accessory cells, was more efficient than that by allogeneic accessory cells. To achieve similar levels of T cell proliferation, in the MLC between $LDAF_1$ T cells and DA T cells, 5 or even 10 fold the numbers of allogeneic responder-strain APCs had to be added to each microwell to effect the same order of proliferation as syngeneic stimulator-strain APCs. The differences in proliferation caused by 10^4 LDA, $5x10^4$ DA, or 10^5 DA accessory cells were not significantly different. All three were significantly different from that caused by 10^4 DA accessory cells ($p<0.001$).

These other contaminating cells were mainly all Class II MHC antigen positive, and were removed by complement lysis, using rabbit complement and an anti-Class II MAb. The suspension was incubated with 14.4.4. and rabbit complement in the following manner:

Having removed all the iron particles, the cells were washed in DMEM and FCS and brought to a final volume of 10 mls. One hundred lambda of the anti-Class II monoclonal antibody, 14.4.4., together with 1 ml of rabbit complement, previously absorbed sequentially on rat erythrocytes and lymphocytes, were added to the cellular suspension and incubated for one hour at 37°C. The contents were washed in *complete* media containing 20% FCS, 1% glutamine, 1% non-essential amino acids, 0.1% Hepes buffer and 1% penicillin, streptomycin and gentamicin. The cells were counted and brought to a final concentration of 5×10^5 cells per ml. Thus, 100 λ of this suspension would contain 5×10^4 cells.

7.3 PREPARATION OF SPLENIC ACCESSORY CELLS

Enriched populations of splenic accessory cells were used as antigen presenting cells. They were prepared using their property of being partially adherent to plastic. A single cell preparation, obtained from a rat spleen, was suspended in DMEM with 10% FCS, as above. The cells were centrifuged at 1200 G for 20 minutes over Histopaque (Sigma, specific gravity 1.077) and the interface collected and washed. This lymphocytic cell suspension was brought up in 40 mls of complete media (see above); 4 x 10 mls were plated into 20 ml petri dishes and incubated at 37°C for two hours.

The dendritic cell population, together with macrophages and other adherent cells, would be adherent to the plastic at this time and the supernatant therefore safely discarded. The "dendritic cells" would remain at the bottom of

FIG. 7.3

MIXED LYMPHOCYTE CULTURE DEMONSTRATING PRETREATMENT OF DONOR WITH ANTI-CLASS II MAB ABROGATES THE STIMULATORY CAPACITY OF SPLENIC ACCESSORY CELLS

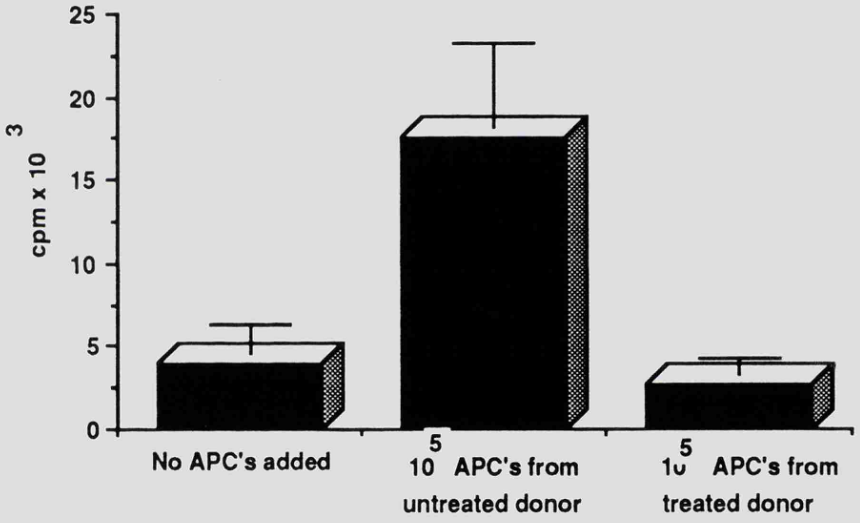

Fig. 7.3 MLC between 10^5 $LDAF_1$ T cells and 10^5 DA T cells. This graph demonstrates the effect on the antigen presenting function of splenic antigen presenting cells (APC's), isolated from a $LDAF_1$ animal pretreated with anti-Class II MAb.

The graph shows that in a T cell - T cell MLC, when the APCs providing accessory cell function are isolated from an animal pretreated with 1 mg of the anti-Class II MAb, 14.4.4., then their accessory function is abolished. The level of inhibition was significantly different from the stimulation caused by APC's from an untreated donor ($p<0.001$).

FIG. 7.4

MIXED LYMPHOCYTE CULTURE DEMONSTRATING IN VITRO PRETREATMENT OF ANTIGEN PRESENTING CELLS INHIBITS THEIR CAPACITY TO STIMULATE T CELL PROLIFERATION

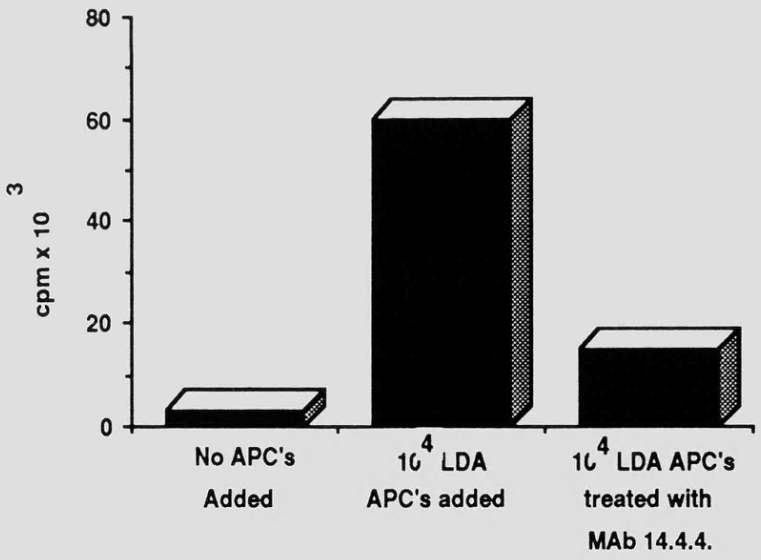

Fig. 7.4 MLC between 10^5 $LDAF_1$ T cells and 10^5 DA T cells. The results demonstrate that adding $LDAF_1$ accessory cells (APC) to the MLC causes a significant stimulation and proliferation of allogeneic T cells. The stimulatory capacity of the APC's, however, are inhibited by *in vitro* pretreatment with the anti-Class II MAb, 14.4.4.

the petri dish. The dishes were therefore washed and the media discarded. Ten mls of fresh media were then added to the petri dishes which were subsequently incubated for a further 18 hours at 37°C. This allowed the dendritic cells, which should have lost their adherent properties at this time, to become freely suspended in the culture media. The macrophages and other adherent cells remained firmly adherent to the walls of the dish for at least another 24 hours. The supernatant, containing primarily dendritic cells, or "loosely adherent splenic accessory cells" was collected and the cells washed and counted. It was these cells that were used in the following experiments as splenic antigen presenting cells.

7.4 MIXED LYMPHOCYTE CULTURES (MLC)

The mixed lymphocyte cultures were set up using $LDAF_1$ splenic T cells as stimulators and DA splenic T cells as responders. The T cells were prepared as above, using complete media (see 7.2), and the stimulator cells irradiated with 2000 rads with gamma irradiation to prevent proliferation. Either 5×10^5 or 2×10^5 stimulator cells were co-cultured with similar numbers of responder strain T cells in flat bottomed multi-well culture plates (Becton Dickinson Labware, Cat No: 1086). 10^5 splenic accessory cells from various strains of rats were irradiated with 2000 rads and added to each well to provide adequate numbers of antigen presenting cells. Cultures were performed in triplicate, under sterile conditions, in 95% O_2 and 5% CO_2 at 37°C for five days. Twenty four hours before harvesting the cells, 1 μCi of tritiated thymidine (Amersham-Seale, Arlington Heights, Il.) was added to each well. On the fifth day of the cultures the cells were harvested and counted in a liquid scintilation spectrometer.

FIG. 7.5

MIXED ISLET LYMPHOCYTE CULTURE DEMONSTRATING THAT PRETREATMENT OF ISLETS ABROGATES THEIR STIMULATORY CAPACITY

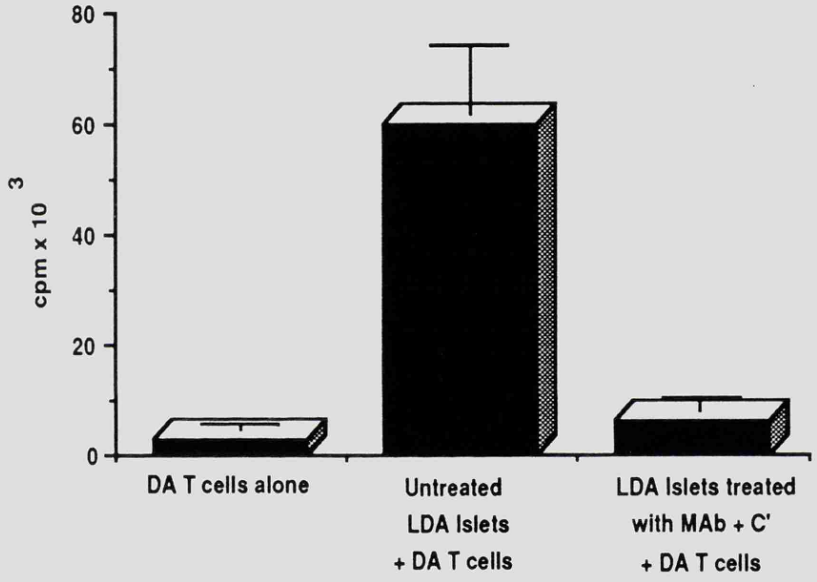

Fig. 7.5 This is a mixed islet lymphocyte culture between 10^5 DA T cells and 150 irradiated LDA pancreatic islets. The effect of pretreating the islets *in vitro* with MAb 14.4.4. and complement (C') is shown.

It can seen that the proliferative response of the T cells is completely abolished after pretreating the allogeneic islets with the anti-Class II MAb ($p<0.0001$).

FIG. 7.6

MIXED ISLET LYMPHOCYTE CULTURE DEMONSTRATING THE EFFECT OF PERFUSING A PANCREAS WITH MAb

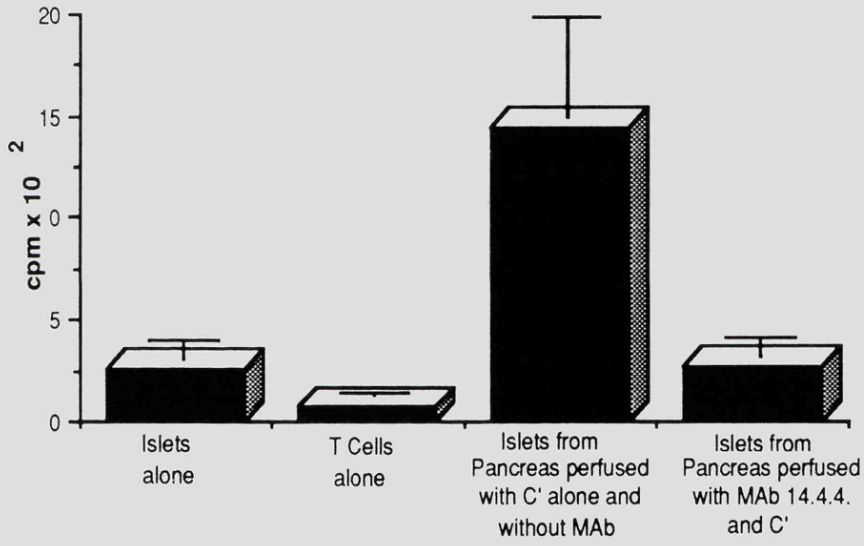

Fig 7.6. This is a mixed islet lymphocyte culture (MILC) between 10^5 DA T cells and 150 $LDAF_1$ islets isolated from perfused pancreases, and compares the stimulatory capacity of islets isolated from pancreases which were perfused with either MAb 14.4.4. and complement (C'), or C' alone.

These results demonstrate that the proliferation of allogeneic DA T cells in response to $LDAF_1$ islets, in a MLIC, was abolished when the islets were isolated from a pancreas pretreated by ex vivo perfusion with an anti-Class II MAb and C', compared to C' alone ($p<0.001$).

7.5 MIXED ISLET LYMPHOCYTE CULTURES (MILC)

The MILC was set up in a manner similar to the MLC. Instead of using $LDAF_1$ splenic T cells as stimulators, 100 hand picked pancreatic islets, with an average diameter of 150 μm, were used and similarly irradiated with 2000 rads. The islets were thus used as stimulators for the allogeneic T cells. The passenger leucocytes, within the islets, were thought to provide enough antigen presenting function for these *in vitro* cultures. The MILC was cultured for five days and on day 4, 1 μCi of tritiated thymidine added to each well. The cells were harvested 18 hours following pulsing with the thymidine and counted as above.

7.6 RESULTS

7.6.1 The use of splenic adherent cells as antigen presenting cells (APC's).

Both Figs 7.1 and 7.2 are graphic results of mixed lymphocyte cultures between 10^5 $LDAF_1$ T cells and 10^5 DA T cells. Fig. 7.1 shows the effect of adding increasing quantities of splenic APCs from DA spleens. It can be seen that the level of proliferation, as measured by tritiated thymidine incorporation is almost linearly dependent on the numbers of APCs added. There was no proliferation of T cells when the APCs were omitted from the microwells. Furthermore, Fig 7.2 compares the effect of adding APCs from either the donor (stimulator) strain or DA (responder) strain animals. In this experiment there was effective stimulation and proliferation of responder T cells when both stimulator strain ($LDAF_1$) and responder strain (DA) accessory cells were added to the mixed lymphocyte cultures. However, the efficacy of stimulation and proliferation, as measured by tritiated thymidine uptake, is significantly different. The 'effective' antigen presentation by the respective APCs differed such that a five fold increase in the numbers of responder strain APCs were necessary to

achieve similar levels of proliferation as syngeneic stimulator strain APCs. 10^4 APCs from the syngeneic stimulator strain $LDAF_1$ produced significant T cell proliferation but the numbers had to be increased to 5 x 10^4 or even 10^5 APCs when they were isolated from the host strain responder animal.

The prerequisite role of the APC in a T cell-T cell MLC is confirmed because when APCs were omitted from the MLC, no stimulation of T cells occurred. Furthermore, the dependent role of the APC is exemplified in Fig. 7.3. This graph compares the function of splenic adherent cells isolated from an animal which has been pretreated with an anti-Class II MAb. Twenty four hours before the APCs were prepared, the animals were injected intravenously with 0.25 ml of 14.4.4. ascites. This corresponded to a dose of 0.5 mg per animal or 2 mg/kg. Higher doses of the MAb killed the animals. As expected, the numbers of adherent cells were significantly reduced, and splenic adherent cells from more than one animal had to used.

Fig. 7.3 shows that the APCs prepared from animals pretreated with MAb did not provide the necessary antigen processing properties in the MLC. It can be seen that the stimulatory and accessory role functions of APCs prepared from a control group of animals, injected with a similar volume of normal saline, remained normal. Pretreating the donor therefore abrogated the ability of it's accessory cells to provide antigen presenting properties.

In contrast to the *in vivo* pretreatment discussed here, in a different experiment, the APCs were first isolated from a spleen and subsequently treated *in vitro* with an anti-Class II MAb, prior to adding them to the MLC. Fig. 7.4 shows the effect of pretreating splenic accessory cells isolated from $LDAF_1$ spleens, for one hour with 14.4.4. and C'. 20 λ of 14.4.4. was added to 10 ml of DMEM containing 10^7 splenic adherent cells, and incubated at 37°C with 1 ml of absorbed rabbit C'. The cells were then washed three times in complete media and again counted. The numbers of splenic accessory cells were significantly reduced and cells from more than one animal were used. Once again the necessary stimulatory function of the APC was abolished when these APCs were pretreated *in vitro* with 14.4.4. and C'.

PLATE 7.1 a

MIXED ISLET LYMPHOCYTE CULTURE

CONTROL ISLETS DAY 2

Plate 7.1a Photomicrograph of a mixed islet lymphocyte culture on DAY 2 between untreated $LDAF_1$ islets and DA T cells. The islets were stained with neutral red.

This photograph shows the pancreatic islets surrounded by small clusters of allogeneic T cells.

PLATE 7.1 b

MIXED ISLET LYMPHOCYTE CULTURE

CONTROL ISLETS DAY 4

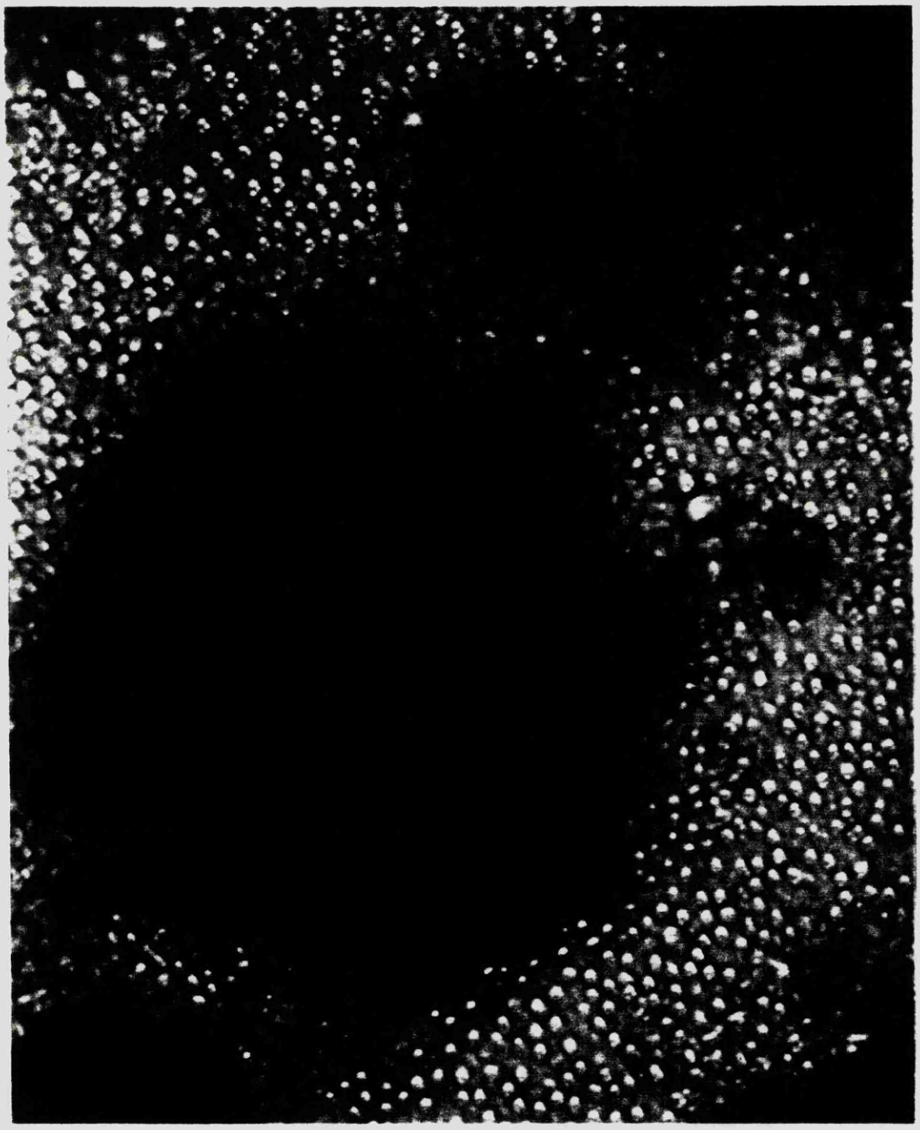

Plate 7.1b Photomicrograph of a mixed islet lymphocyte culture on DAY 4 between untreated $LDAF_1$ islets and DA T cells. The islets were stained with neutral red.

This photograph demonstrates the tremendous proliferation of T cells surrounding the islet. Note that the clear outline of the islet completely obliterated by the presence of proliferating T cells.

PLATE 7.1 c

MIXED ISLET LYMPHOCYTE CULTURE

PRETREATED ISLETS DAY 2

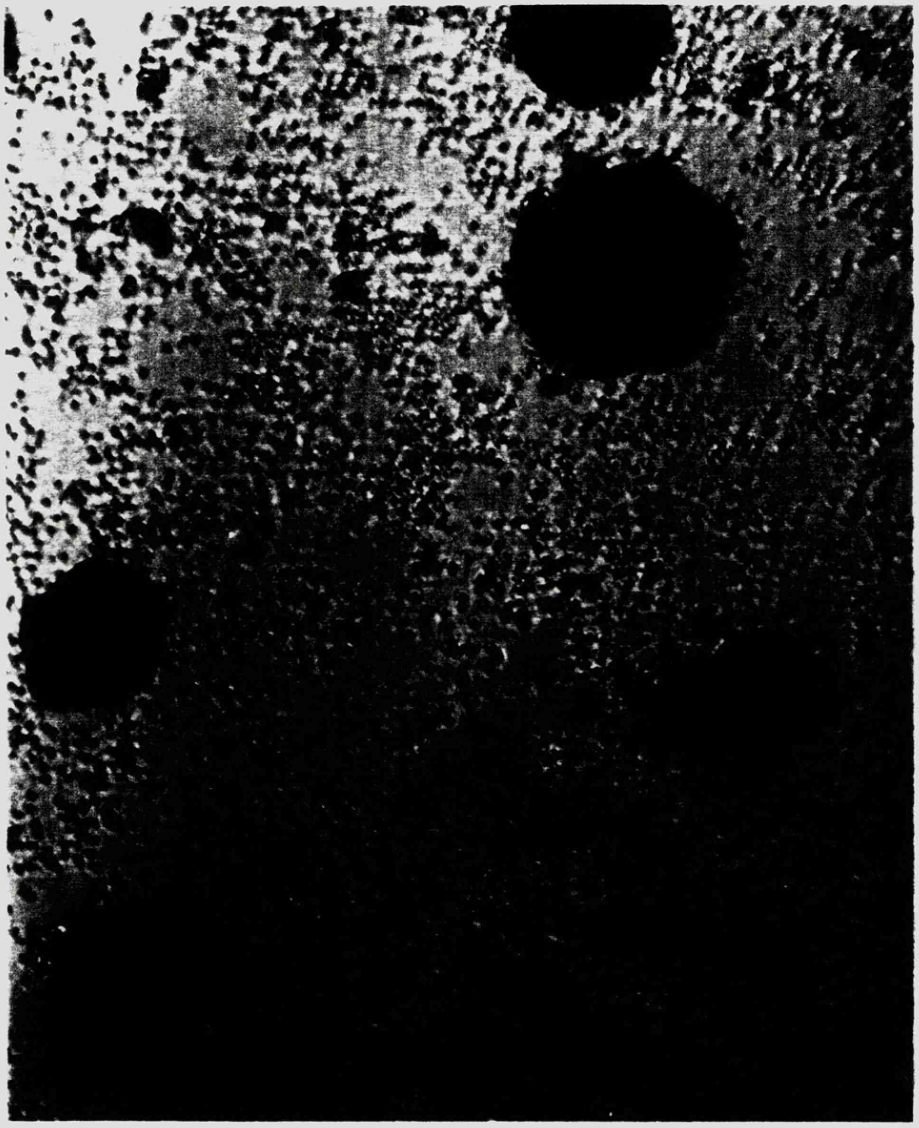

Plate 7.1c Photomicrograph of a mixed islet lymphocyte culture on DAY 2 between $LDAF_1$ islets isolated from pancreases perfused with anti-Class II monoclonal antibodies and DA T cells. The islets were stained with neutral red.

In this MILC there was no significant proliferation of T cells around the pretreated islets, which contrasts to the stimulation and proliferation of T cells surrounding normal untreated islets. (see Plate 7.1a).

PLATE 7.1d

MIXED ISLET LYMPHOCYTE CULTURE
PRETREATED ISLETS DAY 4

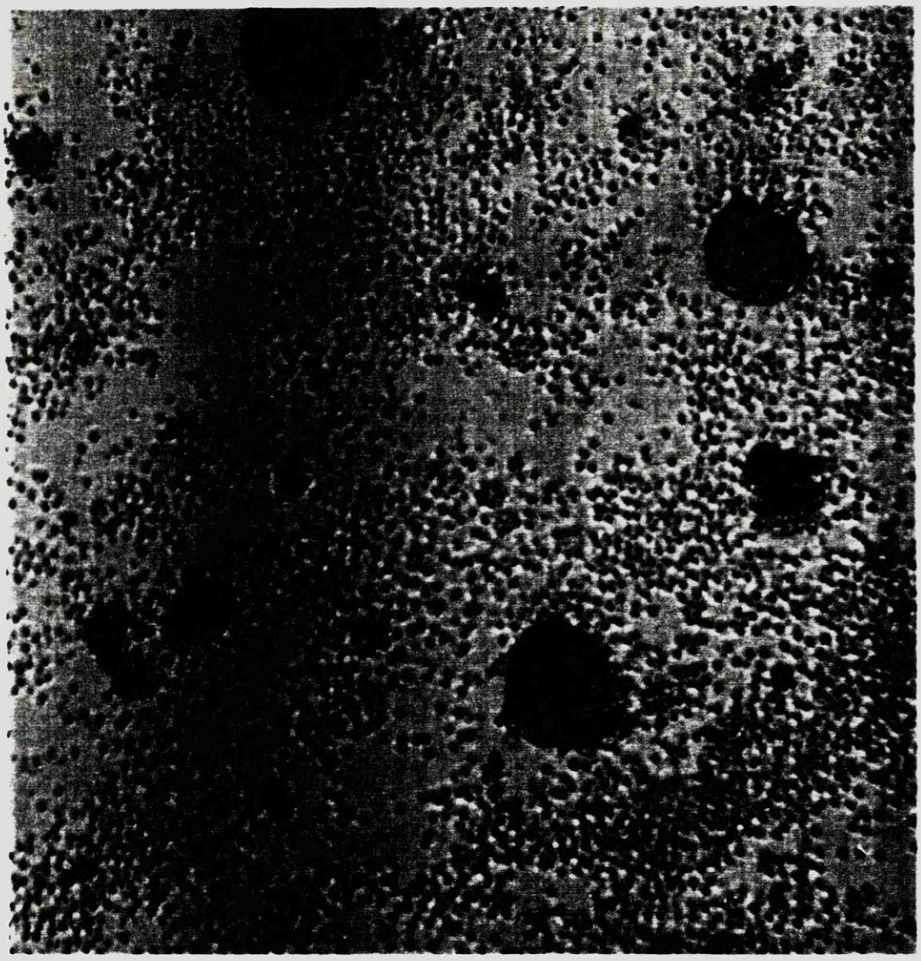

Plate 7.1d Photomicrograph of a mixed islet lymphocyte culture on DAY 4 between $LDAF_1$ islets isolated from pancreases perfused with anti-Class II monoclonal antibody and DA T cells. The islets were stained with neutral red, although the red colour within the islets has faded significantly at this time.

This photograph demonstrates that even after 4 days in culture there was no significant proliferation of T cells around the islets. This contrasts with the gross stimulation and proliferation of T cells produced by control untreated islets as seen in Plate 7.1c.

7.6.2 Effect of Anti-Class II MAb on the stimulatory capacity of pancreatic Islets in the MILC.

Histological sections of pancreatic islets demonstrated a wide variation in the numbers of Class II antigen positive cells contained within them (Plate 7.1). It can be estimated that each rat islet contains between 10 and 50 Ia bearing cells, and that the average islet measuring 100 - 150 μm would contain about 30 or 40 Class II positive passenger cells. Most of these have been identified as Ia bearing non-phagocytic dendritic cells. During a MILC, therefore, in which 100 hand-picked islets were used as stimulators, 3 or 4 x 10^3 Class II positive cells would be present in each microwell. These would potentially provide the antigen presenting properties necessary to drive the MLIC. Previous studies (Fig. 7.1 and Fig 7.2) have shown that 1 x 10^4 allogeneic APCs are sufficient to provide significant proliferation of responder T cells. It proved difficult to add more islets to each well in an effort to increase the numbers of Ia positive cells, as the yield of islets in one particular day were limiting. Nevertheless, 100 islets proved sufficient to cause proliferation of DA T cells (Fig.7.5). In this experiment, the islets were suspended in 4 mls of complete media and incubated at 4°C with 20 λ of 14.4.4. (ascites) for ninety minutes. The islets were washed with complete media three times and subsequently incubated with 0.5 mls of absorbed rabbit C' at 37°c for a further ninety minutes. 100 hand-picked islets were irradiated and placed in microwells with 10^5 DA T cells. The control islets were treated in an identical fashion except 20 λ of normal saline was added instead of the MAb. Control islets were similarly pretreated with C' as in the treatment group. Once again, the *in vitro* pretreatment of these pancreatic islets with MAb and C' inhibited their ability to stimulate allogeneic T cells compared to control islets pretreated with C' alone.

7.6.3 The Effect, in a MILC, on the Stimulatory Capacity of Pancreatic Islets Isolated from Pancreases perfused with MAb and C'.

There is morphological evidence that the Class II bearing cell population is labelled with MAb during ex vivo perfusion (Chapter 5). In the following unique set of experiments, a functional assay was applied to ascertain the immunological effects of perfusing the pancreas with anti-Class II MAb.

Pancreatic islets were isolated from pancreases which were perfused ex vivo for three hours. The method for isolating pancreatic islets had to be adapted. The common bile duct was cannulated with a 23 Gauge Angiocath prior to perfusion, and secured with a 6.0 silk ligature. The pancreas was perfused in the usual fashion allowing the secretions to drain freely into the perfusate. Five minutes before the end of the perfusion period, 1 ml of Neutral Red (concentration 6mg/ml) was added to the perfusate. When the gland had stained sufficiently dark, to facilitate isolation of the islets, the pancreas was removed, flushed with 1 ml of ice-cold saline and distended with 5 mls of collagenase solution as described in Chapter 4. The isolation of the islets then followed the protocol as before. The yields were somewhat decreased as only the left segment of the pancreas was used in the experiments.

Fig. 7.6 shows the effect on the stimulatory capacity of islets removed from pancreases perfused with MAb and C'. The control islets were isolated from pancreases perfused with C' alone. It can be seen once again that 100 hand-picked $LDAF_1$ pancreatic islets, isolated from an organ perfused with C' alone, provided enough additional accessory cell function to provoke stimulation of the allogeneic DA T cells. Moreover, when the pancreatic grafts were perfused ex vivo on the circuit for three hours with both 14.4.4. and C' added to the perfusate, this accessory cell function was abolished; there was no significant stimulation of DA T cells when the islets were isolated from a pancreas perfused with an anti-Class II MAb and C'.

7.7 DISCUSSION

These sets of *in vitro* experiments confirm the importance of Class II MHC antigen bearing splenic accessory cells in the proliferative response of allogeneic T cells. It can be concluded that the presence of Ia-positive, loosely adherent splenic accessory cells, are of paramount importance in the primary allogeneic T cell response in the rat mixed lymphocyte cultures described above. There was minimal autologous stimulation when T cells alone were placed in culture. Similarly, heterolous T cell populations lacked significant background stimulation. It was only the addition of APCs from either stimulator strain or responder strain which provided the stimulus necessary to evoke an immuno-proliferative response.

In each set of experiments, this capacity could be inhibited by the pretreatment with anti-Class II MAb's. This could be accomplished either by *in vitro* pretreatment with 14.4.4. or ex vivo pretreatment of pancreatic islets by perfusion of the whole organ with the anti-Class II MAb's and subsequent isolation of the pancreatic islets.

Of further interest was that the antigen presenting functions of host dendritic cells were less effective in causing proliferation of their syngeneic host T cells as compared to donor-type DC's. This was a consistent finding. In other words, here is confirmation that the two routes for antigenic stimulation proposed by Lechler and Batchelor may infact exist. Moreover, the presentation of allogeneic antigenic determinants to T (probably helper) cells is more effective when presented by the foreign antigen presenting cells. The hypothesis would be that the foreign graft depleted of its antigen presenting cells would have to shed its Class II antigens such that they could be taken up by the host antigen presenting cells, processed and finally presented on the cell surface in such a way as to stimulate a T helper immune response.

Here then, is confirmation that pretreating tissues (cells and islets) with anti-Class II MAb's prior to transplantation, can reduce their ability to evoke an immune response, *in vitro*. If the mixed lymphocyte cultures, and

perhaps the mixed islet lymphocyte cultures, are an *in vitro* equivalents for the initial events occurring in an allogeneic immune response, then the pretreatment of tissues with anti-Class II MAb's could probably reduce the immunogenicity of those tissues, including whole organs.

CHAPTER 8

RICIN

8.1 INTRODUCTION

It was the death of the Bulgarian broadcaster, Georgi Markov, in 1976, apparently by a ricin bullet, which intensified the interest amoungst the scientific community in the application of toxins to the field of medicine (Hughes, 1979). No doubt public awareness increased for sometime thereafter too. Generally, there has been wide interest in utilising the toxic effects of ricin and other toxins, such as abrin, in the hope for site-directed killing of tumours. Moreover, the possibility and success of linking these toxins to a monoclonal antibody has opened a new field in transplant immunology involving specific 'immunotoxin-directed' killing of specific subsets of cells.

Approximately 80 years ago, Paul Ehrlich discussed the potential use of proteins as carriers of pharmacological agents (Himmelweit, 1960). In the 1950's, Pressman suggested that antibody could act as such a carrier (Pressman and Korngold, 1953). He coupled tumour-reactive antibody to isotopes and used these conjugates experimentally to determine the specificity of binding with encouraging results. Most of the currently available antitumour agents have severe dose limiting side effects owing to their detrimental effects on rapidly proliferating normal cells as well as target tumour cells. This problem has been overcome by combining the cytotoxic agents such as alkylating agents, anti-metabolites, antibiotics, enzymes, and diphtheria toxin with anti-tumour immunoglobulins.

During the last decade, there has been considerable progress in the application of this concept to the elimination of cells that are reactive with antibodies coupled to toxic agents. The ability of immunotoxins to kill specific subsets of cells efficiently *in vitro* has led to their application in the deletion of particular cell types in suspensions of bone-marrow cells (Thorpe and Ross,

1982).

A toxin, such as ricin, may be the whole molecule or a polypeptide portion carrying the toxic activity and most of the understanding of the mechanisms by which these toxins kill cells rests on the studies of another toxin isolated from diphtheria (Vitetta and Uhr, 1985). The use of this particular toxin in clinical situations, however, is restricted because of the presence of antibodies to diphtheria toxin and most recent investigators have turned their attention other naturally available (plant) toxins.

Current research has two major objectives: To use these reagents to eradicate cancer and to modulate the host immune response following tissue or organ allografting. However, these *in vivo* treatment regimens still have potential problems which include non-specific toxicity due primarily to damage to the reticuloendothelial system (RES), by deconjugation of the immunogobulin - toxin complex. It is this unpredicted and non-selective destruction of normal cells that has limited the application of immuno-toxins in the clinical field.

In the context of the research presented here, a further application can be envisaged: that of *ex vivo* immunoalteration of vascularised whole organ allografts by *ex vivo* pretreatment with an immuno-conjugate. This would have the obvious advantage that the non-specific damage to the host's immune system could be avoided. McKenzie and colleagues have successfully used ricin - immunoconjugates for the *in vitro* pretreatment of murine bone marrow to deplete T cells, prior to allogeneic transplantation, which also prevented graft versus host disease (GVHD), and have recently used Idarubicin - antibody conjugates to successfully prolong allograft survival in mice (McKenzie *et al,* 1988).

There are relatively few reports of experimental use of immunotoxins *in vivo*. Moolton described the effective use of tumour reactive diphtheria toxin conjugates in an SV-40 induced tumour model (Moolton *et al,* 1975). Krolick and colleagues have reported some success treating BCL_1 leukaemic tumours in mice with anti-idiotype-A chain and anti-δ A chain immunotoxins (Krolick *et al,* 1982).

8.2 RICIN: BIOCHEMISTRY AND MECHANISM OF PENETRATION INTO CELLS

Ricin, is extracted from the castor bean, *Ricinus Communis*, and like most toxic proteins has a toxic polypeptide (A chain) attached to a cell-binding, generally non-toxic polypeptide (B chain). The B chain is a lectin that binds to galactose-containing glycoproteins or glycolipids on the cell surface similar to other toxins, such as abrin and modeccin, which bind to specific cell surface receptors. By mechanisms that are not well understood, ricin A chain gains access to the cell cytoplasm. It is presumed, but not proven, that the route of entry is by receptor-mediated endocytosis, and that the A chain, which has a hydrophobic portion (Boquet *et al,* 1974), penetrates the membrane of an endocytic vesicle or phagosome to enter the cytoplasm (Goldstein *et al,* 1979). Papenheimer has presented compelling evidence for the direct transfer of the toxic principle of diphtheria toxin (Papenheimer, 1977). Diphtheria toxin, a 62K protein, contains hydrophobic peptide sequences in the B region (40K), located at the C-terminal of the whole toxin molecule. Presumably, diphtheria toxin first binds to surface receptors and then inserts into the the phospholipid bilayer by more stable transverse associations mediated through the hydrophobic peptide sequences.

The B chain may have a second role in facilitating entry of the A chain into the cell through the endocytic vesicle (Hughes, 1979). Once in the cytoplasm, the A chain inhibits protein synthesis by enzymatically inactivating the EF2-binding portion of the 60S ribosomal subunit. Since the A chain is non-toxic until it enters the cytoplasm, conjugates of antibody and A chain should be relatively non-toxic to non-phagocytic cells lacking the surface molecules to which the antibody is directed. In addition, the release of A chains from killed cells should not pose a significant problem for "bystander cells."

When the whole toxin (A chain and B chain) is conjugated to an antibody, the now formed 'immunotoxin' can bind cells either by antibody specificity or lectin binding by galactose receptors. Since the binding of the toxin through its B chain is dependent on the presence of galactose receptors, then the presence of high concentrations of lactose or galactose will prevent the lectin

binding by the immunotoxin and thereby leave only its antibody specificity. It is this approach which has been applied in the experiments utilised herein.

It follows that immunotoxins containing chemically derived A chains i.e. ricin A chain, or naturally occuring A chains (gelonin) do not require the use of galactose or lactose since the lectin binding is absent. Optimal toxicity, however, is in some part dependent on the lectin-binding process or its subsequent mechanisms, as toxicity is reduced when A chain is used alone. The mechanisms are not clearly understood but the entry of the active toxic moiety may in fact be optimally facilitated by the binding of the lectin moiety to the galactose receptors. The ease of entry would therefore be reduced following removal of the B chain but returned to some extent by the binding of the antibody. Also, it may be that monoclonal antibodies differ in their ability to elicit endocytosis since F(ab)' fragments are less toxic than $f(ab)'_2$ (Masuho and Hara, 1980). This may indicate the importance of cross linking and endocytosis in cell killing.

8.3 PREPARATION OF IMMUNOTOXINS

The problem for the chemist is to form a covalent linkage between two dissimilar structures, immunoglobin and toxin, to give a stable product of defined composition. Many compounds have been employed including chlorambucil, N-succinimidyl-3-(2-pyridyldithio)propionate (Pharmacia SPDP reagent), and haloacetyl derivatives (Thorpe and Ross, 1982). The ricin-immunoglobin complex used in the following experiments utilised the covalent properties of N-succinimidyl-3- (2-pyridyldithio)propionate (SPDP) to stabilise the immunotoxin. This was a kind gift from Dr. Yoko Mullen, at the University of California, Los Angeles, California. The monoclonal antibody (MAb 13.4) was reactive against pig and rat Class II MHC antigens, and had been shown to deplete Class II positive cells from pig pancreatic islets (Mullen, 1988). Furthermore, the 13.4 MAb-ricin A-chain conjugate had been shown to inhibit rat and mouse mixed lymphocyte cultures by pretreatment of the stimulator cells

FIG. 8.1

PREPARATION OF RICIN IMMUNOTOXIN

Reaction 1:

$$\underset{\text{(IgG)}}{IgG\sim NH_2} + \underset{\text{(SPDP)}}{PySSCH_2CH_2COON(COCH_2)_2} \rightarrow \underset{\text{(IgG-SPDP-conjugate)}}{IgG\sim NHOCCH_2CH_2SSPy}$$

Reaction 2:

$$\underset{\text{(IgG-SPDP-conjugate)}}{IgG\sim NHOCCH_2CH_2SSPy} + DDT \rightarrow \underset{\text{(Thiolated IgG-SPDP-cojugate)}}{IgG\sim NHOCCH_2CH_2SH}$$

Reaction 3:

$$\underset{\text{(Ricin Toxin)}}{Ricin\sim NH_2} + MBS \rightarrow \underset{\text{(Ricin-MBS-complex)}}{Ricin\sim MBS}$$

Reaction 4:

$$\underset{\text{(Thiolated IgG-SPDP)}}{IgG\sim NHOCCH_2CH_2SH} + \underset{\text{(Ricin-MBS-complex)}}{Ricin\sim MBS}$$

$$\downarrow$$

$$\underset{\textbf{(Immunoglobulin-Ricin-Conjugate)}}{IgG\sim NHOCCH_2CH_2SH\text{-}MBS\sim Ricin}$$

Fig. 8.1 The preparation of the Ricin-Immunoglobulin Conjugate. Initially, SPDP (see text) is introduced into the Immunoglobulin (IgG) (Reaction 1), which is subsequently thiolated with dithiothreitol (DTT, Reaction 2). The ricin toxin is derivatised with maleimidobenzoyl succimide (MBS) (Reaction 3). Finally, the thiolated-SPDP-IgG conjugate is incubated with the Ricin-MBS complex to allow formation of the Immunoglobulin-Ricin-Conjugate (Immunotoxin, Reaction 4).

(Nakahara *et al,* 1985).

The use of the SPDP reagent is fully described by Carlsson (Carlsson *et al,* 1978). Essentially one introduces 2-pyridyl disulphide groups into either or both of the proteins as outlined in the reactions described in Fig. 8.1. Basically, 4 moles of pyridyl-2-disulphide groups were incubated at 4°C with 1 mole of antibody (IgG) for 24 hours (Reaction 1). Treatment of the IgG-SPDP product with dithiothreiol (DTT) generates a thiol group, which was desalted on a Sephadex G - 25 column in phosphate buffered saline (PBS, pH 6.5), to form the thiolated-IgG-SPDP product (Reaction 2). The Ricin was derivatised with m-maleimidobenzoyl-N-hydroxy-succimide (MBS) using a method described by Youle and Neville (Reaction 2)(Neville and Youle, 1982). Incubation of ricin at 2 mg/ml with a ten fold MBS molar excess was carried out for 30 mins at 25°C followed by gel filtration on a G-25 column equilibrated in PBS (pH 6.5) to remove unreacted MBS. For conjugation, the thiolated antibody (thiolated-IgG) at 3.1 μM was mixed with 9.8 μM ricin-MBS. The reaction was allowed to proceed for 1 hour at 25°C, then for 18 hr at 4°C (Reaction 4). The immunotoxin thus formed was purified by high pressure liquid chromatography (HPLC) utilising gel permeation on a TSK-259 column (Bjorn *et al,* 1985).

TABLE 8.1

EFFICACY OF CLASS II POSITIVE CELL LABELLING WITH RICIN IMMUNOTOXIN

Rat Strain	Monoclonal Antibody	
	14.4.4.	13.4-Ricin
$LDAF_1$	72 ± 16	61 ± 11

Table 8.1 Table showing the numbers of Class II antigen positive cells / mm^2 labelled on $LDAF_1$ pancreases during a two hour ex vivo perfusion with either 14.4.4. or the ricin-conjugate, 13.4-Ricin. It can be seen that similar numbers were labelled (the difference in the numbers were not significant, $p>0.5$), indicating that the ricin conjugate MAb labels all Class II positive cells in the rat pancreas.

TABLE 8.2

SURVIVAL OF PANCREATIC ALLOGRAFTS PERFUSED WITH RICIN IMMUNOTOXIN

Additive	Number of Animals	Survival (in days)	Deaths
6 hr perfusion			
Galactose Alone	3	0, 0, 0	3
4 hr perfusion			
Galactose Alone	7	7, 9, 12	4
Galactose + 5 λ 13.4-Ricin	7	12, 13, 16, 16	3
Galactose + 20 λ 13.4-Ricin	8	5, 18, 20	5

Table 8.2 Comparison of the effects on pancreatic allograft survival following 4 or 6 hr ex vivo perfusion with galactose and Ricin immuno-toxin, 13.4-Ricin. There were three deaths in the 6 hr perfusion group, and the perfusion period was reduced to 4 hr. The high incidence of animal deaths occuring in the early post operative period (24 - 48 hours) was due to hyperosmolar damage to the pancreas caused by the galactose added to the perfusate. The pancreatic grafts which functioned successfully following perfusion with the 13.4-Ricin immunotoxin had a prolonged allograft survival (see text).

8.4 THE USE OF RICIN CONJUGATED MAb TO DEPLETE CLASS II POSITIVE CELLS FROM THE PANCREAS DURING EX VIVO PERFUSION

8.4.1 Outline

An experiment was performed whereby intact rat pancreases were perfused on the ex vivo circuit with a Ricin-MAb conjugate added to the perfusate. The monoclonal antibody was reactive against rat Class II MHC antigen, and the non-specific binding of the lectin groups to the galactose receptors was inhibited by adding galactose to the perfusate to block the receptors before the addition of ricin. The pancreases were both examined, using the histochemical techniques described in Chapter 6, to analyse the efficacy of labelling of the Class II positive cells within the pancreas with this immuno-conjugate and also, a series of pancreases were perfused for prolonged periods to allow sufficient time for the incorporation of the toxic moiety into the cells following binding with the antibody. These pancreases were transplanted into allogeneic diabetic recipients, to ascertain the effect on survival of Class II positive cell depletion by perfusion with ricin-MAb conjugate.

8.4.2 Method

Initially, a 20 Molar solution of galactose was obtained by dissolving 684 mg of crystalline galactose (Sigma) in 500 mls of Plasmalyte (Travenol, Deerfield, IL.). One ml of this solution was added to 19 mls of the usual perfusate, containing buffy-coat depleted heparinised blood and verapamil (Chapter 5). The final galactose concentration was 1 Molar.

The ex vivo perfusion circuit was set up in the usual way, but great care was taken to avoid skin contact with the ricin conjugate itself and to avoid contacting instruments and laboratory equipment contaminated with it. The

FIG. 8.2

SURVIVAL OF PANCREATIC ALLOGRAFTS PERFUSED WITH RICIN IMMUNOTOXIN

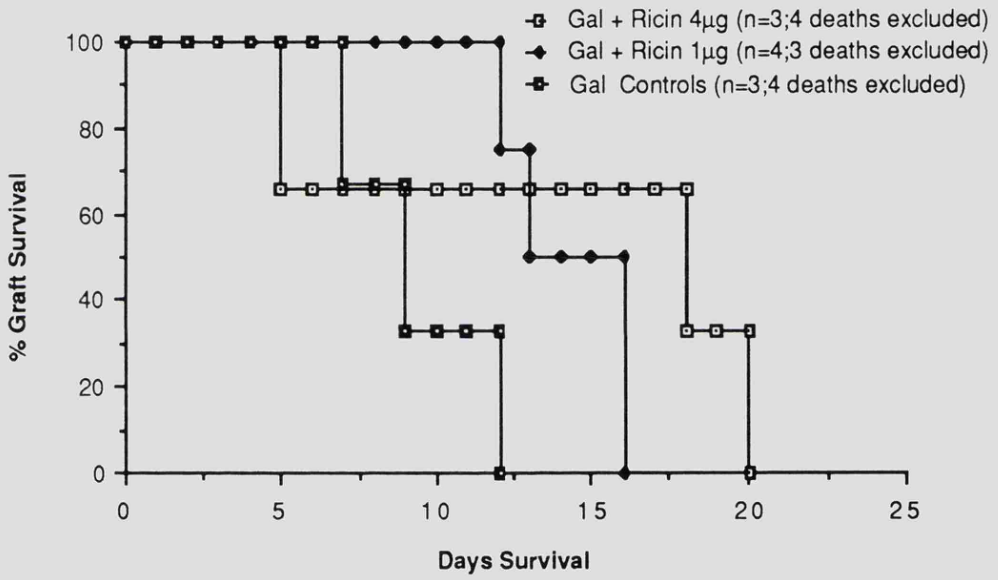

Fig. 8.2 The effect of perfusing pancreases with 1 M galactose and Ricin immunotoxin on the survival of $LDAF_1$ to DA pancreatic allografts.

This graph shows that when the pancreas allografts survived the extended (4 hrs) ex vivo perfusion, there was a slight prolongation of allograft survival when 1 μg or 4 μg of the Ricin-conjugate and galactose was added to the perfusate, compared to the control group in which only galactose was added.

FIG. 8.3

SURVIVAL OF PANCREATIC ALLOGRAFTS PERFUSED WITH RICIN IMMUNOTOXIN

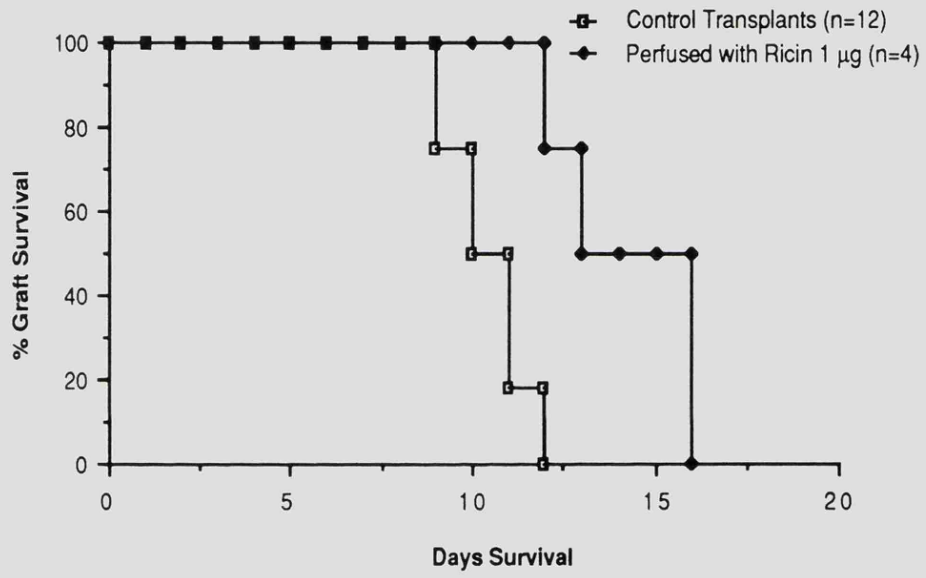

Fig. 8.3 The effect of perfusing pancreases with an immunotoxin, Ricin, conjugated to an anti-Class II MAb, 13.4 on the survival of $LDAF_1$ to DA allografts.

This graph shows that a prolongation of pancreatic allograft survival was achieved when the pancreases were perfused for 4 hrs with an anti-Class II MAb conjugated to ricin toxin, compared to controls, although because of the small numbers the effect was not statistically significant.

ricin-conjugate used was extremely dangerous; indeed, 1 mg of purified ricin would be enough to kill 4000 guinea pigs! (personal communication, Yoko Mullen). This being the case, extra precautions to avoid ricin contact were taken. A face-mask and double gloves were always used when handling this toxin.

To demonstrate adequate labelling of the Class II cells in the animals used in the experiments, pancreases were first perfused for two hours with buffy coat depleted blood containing either 5 λ or 20 λ of the 13.4-Ricin solution. The 13.4-Ricin immunotoxin was dissolved in PBS at a MAb content of 200 μg/ml. i.e. 5 λ contained 1 μg and 20 λ contained 4 μg of ricin-MAb conjugate. The pressure-flow characteristics were reassessed, as the addition of galactose increased the osmolality of the perfusate and interfered with the delicate perfusion dynamics. Following perfusion, as with the non-ricin perfused pancreases, these grafts were flushed with ice-cold saline. However, in these cases 10 mls of saline were delivered through a Hutchinson pump (American Scientific Supply, Chicago, IL., USA) at a rate of 0.1 ml/minute to ensure adequate flushing of the ricin toxin. The pancreases were snap frozen in liquid nitrogen and studied with immunohistochemical probes, as before, to define the numbers of Class II +ve cells labelled with the 13.4 MAb during the perfusion period.

In the second series of experiments, the pancreases were isolated for transplantation. Initially, $LDAF_1$ pancreases were perfused on the circuit for six hours, and subsequently, for four hours prior to transplantation into diabetic DA recipients. The survival of these ricin pretreated pancreatic allografts were monitored by daily assessment of the blood glucose levels.

8.5 RESULTS

The results of the immunohistochemical staining confirmed that Class II positive cells were certainly identified in pancreases perfused with the ricin-conjugate. The results shown in Table 8.1 confirm the numbers of Ia +ve cells that were labelled with 13.4 - Ricin conjugate (61 ± 11 Ia +ve cells/mm^2), were not statistically significant ($p>0.5$) from the numbers of Ia +ve cells

labelled on control $LDAF_1$ pancreases perfused with 14.4.4. for a similar period (72 ± 16 cells/mm^2). The MAb, 13.4 was thus confirmed not only to have retained it's antigenic activity following the coupling with the toxin, but also that it reacted with all the Class II MHC determinants expressed in the $LDAF_1$ rats used in these experiments.

In the second series of experiments, when pancreases were transplanted into allogeneic diabetic recipients, there was a high incidence of graft failure, probably due to perfusion injury. Autopsy verified that even the control pancreases perfused with galactose alone developed gross fibrotic changes. Indeed, 4 of the 7 pancreases failed to function in the control group and the animals died presumably because of the profound effects of hypoglycaemia due to sequestration of insulin released from the damaged pancreas following perfusion with galactose. The 3 animals which had functioning allografts, despite probable perfusion injury, reversed the experimentally induced diabetes in the DA recipients for 7, 9, and 12 days, respectively, mean survival 9.3 days $\pm$ 2.5 days (Table 8.2).

In the experimental groups perfused with the MAb-Ricin conjugate, a similar number of deaths was observed (8 animals out of 15). Again the hyperosmolar damage to the pancreas was thought to be the prime reason for failure but ricin induced death was a consideration. Even though the pancreatic grafts were flushed with 10 mls of saline following ricin perfusion, the extreme potency of the small amount of toxin residual in the pancreas may have been enough to cause death upon revascularisation of the graft in the recipient. Because a ricin assay was not available this remained unanswered, but since there were deaths in the galactose only group, it was thought unlikely.

Despite these graft failures, there were 7 pancreatic grafts in the ricin-perfused groups which functioned for some time following transplantation. Three pancreases perfused with 20 λ (4μg) of 13.4-Ricin functioned well and maintained normoglycaemia in the recipients for 5, 18 and 20 days, respectfully, mean survival 14.3 ± 8.0 days. Similarly, in the 5 λ (1μg) perfusion group, the 4 survivors enjoyed normoglycaemia for 12, 13, 16 and 16 days (mean survival

14.25 ± 2 days) before failure of the graft resulted in hyperglycaemia (figs. 8.2 and 8.3).

It seemed, therefore, that there was a prolongation in allograft survival when pancreases were perfused with the anti-Class II MAb-toxin conjugate on the perfusion circuit. Because the numbers of survivors were small, the difference in allograft survival was not statistically significantly different from control untreated pancreas transplants.

8.6 DISCUSSION

There is great potential for the application of immunotoxins to the field of medicine. These preliminary experiments have shown that the 13.4-Ricin conjugate can be used safely in *ex vivo* experiments to attempt immunoalteration of intact organs by depleting Class II passenger leucocytes. The difficulties incurred using galactose in the perfusate could be overcome.

First, there are now reports that the 1 M galactose solution used to inhibit the binding of the lectin (ricin B chain) can be replaced by a solution with 0.1 M concentration (Nakamara *et al,* 1985; Mullen, 1988). Second, the development of immunotoxins conjugated to only the A chain eliminates the need for galactose inhibition altogether. That the toxic A chain is less efficient at killing when isolated from its B chain has concerned some investigators (Blythman *et al,* 1981). Thorpe and Ross reported successful linking of the A chain of ricin to a murine MAb, W3/25, which recognises an antigen expressed by rat T helper cells (Thorpe and Ross, 1982). However, the conjugate was found not to inhibit the capacity of rat spleen cells to respond in tissue culture to phytohaemagglutinin, a mitogen which predominately stimulates the W3/25-expressing T cell subpopulation. Similarly, Chang and his colleagues, found no killing of mammary cells by a diphtheria toxin A subunit - conjugated to human placental lactogen (Chang *et al,* 1977).

The actual loss of toxicity is presumably due to difficulty in gaining access to the cell cytoplasm. Perhaps, if the hydrophobic portion (B chain) was

replaced by an alternative molecule or protein which could just as efficiently bind to the cell surface such as to ensure penetration into the cell, then the toxicity of the A chain could be maintained. Although there is evidence that the toxicity of the ricin is significantly reduced when isolated from it's B chain, there are encouraging data to support that it still may be of clinical benefit. Other reports have shown that diphtheria A subunit coupled with concavalin A (Gilliland *et al,* 1978), *Wisteria floribunda* lectin (Uchida *et al,* 1978), and human chorionic gonadotrophin (Oeltmann and Heath, 1979) are infact quite cytotoxic and could be used for both *in vitro* and *in vivo* experimentation. Presumably the processing of the conjugates is mediated after binding of the surface binding ligands and leads to penetration of the toxic A subunits.

As mentioned earlier, Nakahara clearly demonstrated that pretreatment of stimulator spleen cells with whole ricin-anti-Class II MAb immunoconjugate abrogated the proliferation of allogeneic rat and mouse spleen cells (Nakahara *et al,* 1985). Now, Wiley *et al* have shown similar success using ricin-A chain conjugated to a rat anti-Class II MAb, OX4 (Wiley *et al,* 1988). They pretreated pancreatic islets from male AGUS x WAG F_1 hybrids ($RT1^{lxu}$) with the ricin A chain-OX4 immunotoxin and demonstrated complete inhibition of their stimulatory capacity in a mixed islet lymphocyte culture with female DA splenocytes. In the animal experiments presented here, pretreating $LDAF_1$ pancreatic islets with ricin-anti-Class II immunotoxin inhibited their capacity to stimulate allogeneic DA T cells.

In summary, the use of ricin and other toxins, whether used as the complete molecule or simply the toxic subunit, has potentially a significant application to the immunomodulation of transplantable tissues. Ricin conjugated to anti-Class II monoclonal antibodies depletes Class II MHC antigen bearing cells *in vitro*, and pretreatment of pancreatic islets with this immunotoxin conjugate abrogates an allogeneic T cell proliferative response. The data presented in this thesis, although preliminary, would support that whole organ graft immunogenicity could be reduced by such measures too.

CHAPTER 9

PANCREATIC ISLETS

9.1 INTRODUCTION

It has already been established that the immunogenicity of pancreatic islets is significantly more than that of pancreatic whole grafts, in certain animal strain combinations (Schulak *et al,* 1984; Groth, 1988). To illucidate the effects of anti-Class II MAb pretreatment on transplantable tissue, pancreatic islets were subjected to anti-Class II pretreatment and the effects on their survival as allografts was determined. The prolongation of survival, if any, could be compared to that of whole pancreas allografts.

A full description of the technique used to isolate islets from the rat pancreas is given in Chapter 4.5. The studies in this Chapter were designed not only to compare the effects of immunological manipulation of islets to those of whole pancreatic allografts, by the pretreatment with MAb's, but to also attempt to study the viability of the pretreated islets after culturing or ex vivo perfusion, by determining the insulin producing ability of the pretreated islets to an *in vitro* glucose challenge during *perifusion.*

9.2 ANTI-CLASS II MONOCLONAL ANTIBODY PRETREATMENT OF PANCREATIC ISLETS.

9.2.1 The Effect of Pretreating Isolated Pancreatic Islets with Anti-Class MAb's on the Survival of Allografts.

Pancreatic islets were isolated from $LDAF_1$ rats and pretreated with 14.4.4. and rabbit complement. Between 750 and 1000 islets were hand picked and suspended in 4 mls of Dubecco's Minimal Essential Media (DMEM, Gibco)

containing 5 % fetal calf serum (FCS). In the MAb treated group, 20 λ of 14.4.4. ascites was added to this suspension which was incubated for 90 mins at 4°C. The tube was shaken intermittently to ensure thorough mixing of the MAb. The islets were gently washed three times with DMEM, containing 5 % FCS, and brought to a volume of 9 mls. Subsequently, they were incubated with 1 ml of rabbit complement for 45 mins at 37°C, bringing the final volume to 10 mls. Finally, the islets were washed again and suspended in 1 ml of normal saline, containing 1 % FCS. This suspension of islets was injected into the portal vein of diabetic DA recipients and blood glucose levels measured daily thereafter. The control group of animals were given islets pretreated with C' alone and no MAb.

Table 9.1 shows the effect of anti-Class II MAb pretreatment on the survival of $LDAF_1$ pancreatic islet allografts. It can be seen that untreated islets were rejected between 7 and 15 days with a mean survival of 10.6 ± 3.1 days (Group 1, n = 8). When islets were pretreated with C' alone for 45 mins, the islets were rejected between 5 and 8 days with a mean survival of 6.8 ± 1.0 days (Group 2, n = 7). The survival of pancreatic islets pretreated with both anti-Class II MAb and C' ranged from 10 to 15 days with a mean of 12.4 ± 2.0 days (Group 3, n = 5).

The difference in survival of control untreated islets compared to the survival of control islets treated with C' alone was suprisingly high. It was statistically significant ($p<0.005$). It emphasises the fact that there is variability in the survival of the islet allografts in this particular strain combination, and demonstrates that the survival can be affected by the actual batch of the animals selected. The experiments in the untreated control group were performed in a different batch of $LDAF_1$ to DA animals, than the experiments performed with C' and MAb. Perhaps there were slight differences in the immunogenicity of the 'historical' group, and direct comparison to the C' treated group could be considered invalid. Nevertheless, even when the mean survival time of this 'historical' untreated group (Group 1) was compared to that of islets pretreated with MAb and C' (Group 3), the prolongation of survival remained highly significant ($P<0.0001$).

TABLE 9.1

THE EFFECT OF PRETREATING PANCREATIC ISLETS WITH MAb AND C' UPON ALLOGRAFT SURVIVAL

Group	No. of Animals	Pretreatment Protocol	Survival (Days)	Mean ± SD (Days)
Islets 1	8	Straight Transplant *(No Pretreatment)*	7, 7, 8, 10, 11, 13, 14, 15	10.6 ± 3.1
Islets 2	7	C' for 45 mins	5, 6, 7, 7, 7, 8, 8	6.8 ± 1.0
Islets 3	5	MAb for 90 mins C' for 45 mins	10, 11, 12, 14, 15	12.4 ± 2.0

Table 9.1 The effect of pretreating $LDAF_1$ pancreatic islets with anti-Class II monoclonal antibody (MAb) 14.4.4. and rabbit complement (C'), on the survival of an intraportal injection of allografts (DA recipients).

This table shows that untreated islets were rejected at 10.6 ± 3.1 days (Group 1). When islets were pretreated with MAb and C', in vitro, there was a significant prolongation in their allograft survival (12.4 ± 2.0 days, Group 3), compared to Group 1 untreated islets ($P<0.0001$) or islets treated with C' alone (Group 2, $p<0.0007$). Suprisingly, there was a reduction of allograft survival when islets were pretreated with C' alone ($p<0.005$).

TABLE 9.2

THE EFFECT OF CULTURING ISLETS WITH MAb AND C' FOR 24 HOURS ON ALLOGRAFT SURVIVAL

Group	No. of Animals	Additive to 24 hr culture medium	Survival (Days)	Mean ± SD (Days)
Islets 4	4	none	5, 6, 6, 13	7.5 ± 3.6
Islets 5	5	C' only	6, 6, 7, 14, 20	10.6 ± 6.2
Islets 6	9	C' and MAb	7, 9, 9, 10, 10, 10, 12, 13, 27	11.8 ± 5.9

Table 9.2 The effect of overnight culture of pancreatic islets with anti-Class II MAb (14.4.4.) and complement (C') on the survival of $LDAF_1$ to DA allografts. This table shows that when islets are pretreated for 24 hrs with MAb and C', there is a significant allograft prolongation compared to untreated controls (11.8 ± 5.9 vs. 7.5 ± 3.6 days, respectively) but that a similar effect could occur with C' alone (10.6 ± 6.2 days). See text for explanation.

Thus, the pretreatment of islets with MAb and C' resulted in statistically significant prolongation of allograft survival whichever two groups were compared. Since criticism could be given to comparing groups of animals transplanted at different times, the isolation, pretreatment and transplantation of the islets in groups 2 and 3 were performed concurrently. Islets were removed from animals shipped in the same batch from the vendor. Following isolation, the islets were randomised either to be pretreated with MAb and C', or pretreated with C' alone. The allograft survival times in this experiment confirmed that pretreatment of pancreatic islets with anti-Class II MAb and C' resulted in significant prolongation ($p<0.0007$). There was indeed a significant prolongation of survival (mean prolongation, 6 days) when pancreatic islets were pretreated with anti-Class II MAb and complement in the strain combination of animals used (see Table 9.1).

9.2.2 The Effect of Overnight Culturing at 37°C with Anti-Class II MAb's and C' on the Survival of Pancreatic Allografts.

Faustman's paper had shown that in a murine model, the pretreatment of islets with MAb and C' resulted in indefinite survival when transplanted into untreated non-immunosuppressed allogeneic hosts (Faustman *et al,* 1984). If total depletion of the Class II antigen bearing cells within the islets was obligatory for optimal immunoalteration, then perhaps the short term pretreatment methods used in the above experiment were suboptimal and a few Class II positive dendritic cells remained functionally intact. Indeed, when islets, pretreated with 14.4.4. and complement for a total of 2 hours, were subjected to immunohistochemical analysis for residual Class II positive cells, it was evident some remained.

To increase the possibility of *in vitro* lysis of these Ia positive cells mediated by complement fixation, the islets were exposed to MAb and C' for prolonged periods. Groups of islets were cultured for 18 - 24 hours, at 37°C in

PLATE 9.1

PANCREATIC ISLET DEMONSTRATING PRESENCE OF A CLASS II POSITIVE CELL AFTER 24 HOURS CULTURE WITH ANTI-CLASS II MAb

Plate 9.1 A photomicrograph showing part of an $LDAF_1$ pancreatic islet demonstrating the presence of two adjacent Class II antigen positive cells (stained black), despite 24 hour culture at 37°C with anti-Class II MAb (14.4.4.) and C'. (Courtesy of Martin Weiser).

Dubecco's Minimal Essential Medium (DMEM) supplemented with glutamine, non-essential amino acids, fetal calf serum, penicillin and streptomycin, in an atmosphere containing 95 % O_2 and 5 % CO_2. The effect of culturing various groups of islets with either 14.4.4. and C', C' alone, or no additives at all, was observed on the survival of allografts. In these experiments, one ml of absorbed rabbit complement was added to 9 mls of culture medium containing the islets which were incubated overnight in a Petri dish. The results are shown in Table 9.2.

Following the transplantation of the islets that were cultured overnight in media alone, allograft survival ranged from 5 to 13 days, with a mean of 7.5 ± 3.6 days (Group 4, n = 4). When C' alone was added to the culture medium, grafts were rejected between 6 and 20 days, mean 10.6 ± 6.2 days (Group 5, n = 5). The prolongation of allograft survival caused by the addition of C' was not that statistically significant ($P<0.05$), although the slight prolongation demonstrated the great variability of the survival of these islet allografts. When islets were pretreated with MAb and C' overnight, there was a similar variation of graft prolongation. These particular islets were rejected between 7 and 27 days with a mean of 11.8 ± 5.9 days (Group 6, n = 9). The prolongation of graft survival in this group was not significantly different from that achieved by C' alone ($p > 0.6$) or even media alone ($p > 0.4$).

The degree of variability of graft prolongation precludes any strong conclusions being drawn from the above data. The numbers of animal experiments in each group would have had to be increased. An explanation for the effect of C' on the prolongation of pancreatic allografts could be that the rabbit complement used contained naturally occuring alloantigens to rat tissue. Even though the C' was sequentially absorbed on rat erythrocytes and lymphocytes, there may have still been enough alloantigens present to cause an immunological effect. This effect was not seen in the whole pancreas allografts perfused with C' presumably because the amount of pancreatic tissue absorbed the antibodies with relatively little reduction in their immunogenicity.

Furthermore, not all the Class II MHC antigen bearing cells may have been eliminated. Indeed, immunohistochemical analysis of islets cultured for 24 hours with MAb and C' revealed some residual Class II bearing cells. Plate 9.1

FIG. 9.1

A SCHEMATIC DIAGRAM OF PERIFUSION APPARATUS

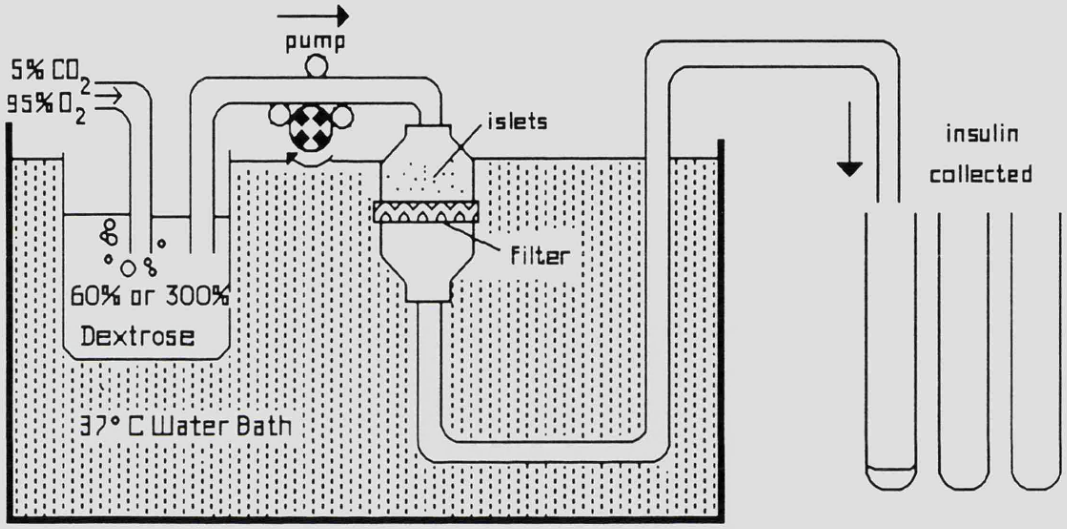

Fig. 9.1 Schematic diagram of the perifusion apparatus used to assess the insulin response of pancreatic islets to a glucose challenge. The islets are placed in a Millipore filter and 60% or 300% Dextrose solution perfused through the filter at a rate of 1 ml/min. Oxygen and carbon dioxide is bubbled through the dextrose solution to maintain a constant pH of 7.4, and the whole apparatus is placed in a 37°C water bath. The insulin produced by the islets in response to the glucose challenge is collected at 5 minute intervals on a fraction collector.

demonstrates that elimination of all Class II positive cells from $LDAF_1$ pancreatic islets was incomplete, even after 24 hours of pretreatment.

We can surmise, however, that an analysis of all the above data suggests that in the particular strain combination used ($LDAF_1$ to DA) immunomodulation is possible, particularly by pretreatment with anti-Class II MAb's and C', despite evidence that total elimination of all the dendritic cells was not achieved. Nevertheless, following transplantation, significant prolongation of allograft survival was achieved though indefinite allograft survival in this strain combination was not seen.

9.3 PERIFUSION STUDIES

9.3.1 Introduction and Methodology

The assessment of pancreatic islet viability can be morphological or functional. The ideal test would of course be a functional one which simulates the *in vivo* situation. The ultimate criterion would be the transplantation of the islets into a syngeneic diabetic recipient to investigate their ability to maintain euglycaemia under physiological conditions. Even though this is the most obvious and perhaps the most important parameter of islet viability, following the various isolation techniques and immunological manipulations, the results are rather crude. There is no definitive way to quantify relatively small changes in islet viability by *in vivo* methods, particularly if only a percentage of the islets were functionally impaired. The glucose would essentially be either controlled (normoglycaemia) or uncontrolled (hyperglycaemia) in the recipients.

Static measurements whereby islets are incubated with or without glucose with subsequent measurement of the total insulin *extracted* from the total number of islets used, would give some indication of viability. However, even this technique is rather crude and gives no indication of the functional response of the islets to a glucose challenge. A dynamic test, therefore, involving the insulin response of isolated pancreatic islets, to 'perfusion' with a high glucose solution, would be more physiological and representative of the *in vivo* situation..

A *perifusion* system was thus developed to study the effects of challenging a group of islets, *in vitro*, to a high, but physiological, glucose load. A schematic diagram of the perifusion circuit is shown in Fig. 9.1. Basically, the circuit consisted of silastic tubing (American Scientific Supplies, Cat. No. 602-285, internal diameter 0.062 inches), a Millipore filter and filter holder (Millipore Labs, 13 mm internal diameter) and a non-peristaltic pump (Gibco, Madison, Wisconsin, USA). 150 pancreatic islets were placed carefully in the proximal side of the filter and were perifused with two glucose solutions.

The first was a physiological solution containing sodium chloride, sodium bicarbonate, magnesium chloride and calcium chloride with 60% D-glucose (see Table 9.1 for biochemical formula). This solution did not stimulate the islets to produce insulin, and was designed to standardise the initial perifusion environment before the glucose challenge.

After 40 mins of perifusing the islets with this low dextrose perfusate, the solution was exchanged for one containing 300% D-glucose. This high glucose concentration in the perifusion fluid caused the release of insulin from insulin granules from within the β cells and also stimulated the neosynthesis of insulin in these cells. The flow of the glucose solution was set at a constant rate of 1 ml per minute, and the pH maintained at 7.4 by bubbling 95% O_2 and 5% CO_2 into the glucose solution reservoirs. The perifusate containing any insulin which may have been released was collected at 5 minute intervals in a automated fraction collector. These samples were frozen and subsequently analysed by a radioimmune assay for insulin concentration. This was kindly performed by Mr. Bill Pugh, Department of Diabetology.

A schematic graph of the 'expected' response if healthy fresh islets were challenged to this high glucose load is given in Fig. 9.2. The response curve has three important phases. After a suitable lag phase which represents the time taken for the high glucose perifusate to pass along the tubing, there is an immediate and almost abrupt rise in insulin output (phase 1). This represents the insulin released from the intracellular stores of the islet β cells. This initial peak starts to decline when all the insulin granules have released their stored insulin. Second, a few minutes after the initial peak another peak of insulin output occurs which reaches a continuous maximum level of insulin release; this is the plateau phase (phase 2).

It represents the amount of insulin newly synthesised in response to the glucose load. This level of insulin remains constant, providing enough energy and metabolic precursors are available, throughout the glucose challenge. When the challenge is removed, there is a corresponding cessation of insulin secretion, represented by phase 3 in Fig. 9.2. In an ideal situation all 3 phases of insulin secretion are present.

If there is functional impairment to the islets, there would be abnormalities in one or more of the phases of insulin release. Healthy islets, devoid of stored insulin, would lack phase 1, but would be able to synthesise insulin in response to a glucose challenge; consequently phase 2 would be normal. Damage causing impairment of protein synthesis would cause a dimunition or inhibition of the insulin levels in plateau phase 2, and abnormalities in phase 3 would represent poor functional control as the islets could not 'switch off' their insulin production in response to a low glucose challenge.

9.4 RESULTS

9.4.1 Perifusion of Freshly Isolated Pancreatic Islets

Figs. 9.3 and 9.4 demonstrate that the response curves of freshly isolated pancreatic islets is analogous to that of ideal islets as depicted in Fig. 9.2. 150 islets were challenged in the perifusion apparatus and produced insulin in response to the glucose challenge. Not only was there an early degranulation (Phase 1) but the viability of the islets was demonstrated by the second peak or plateau phase (Phase 2). Furthermore, there was a normal 'cut off' of insulin secretion when the glucose challenge was removed.

FIG. 9.2

A SCHEMATIC GRAPH OF THE INSULIN RESPONSE OF 'PERFECT' ISLETS TO A GLUCOSE CHALLENGE

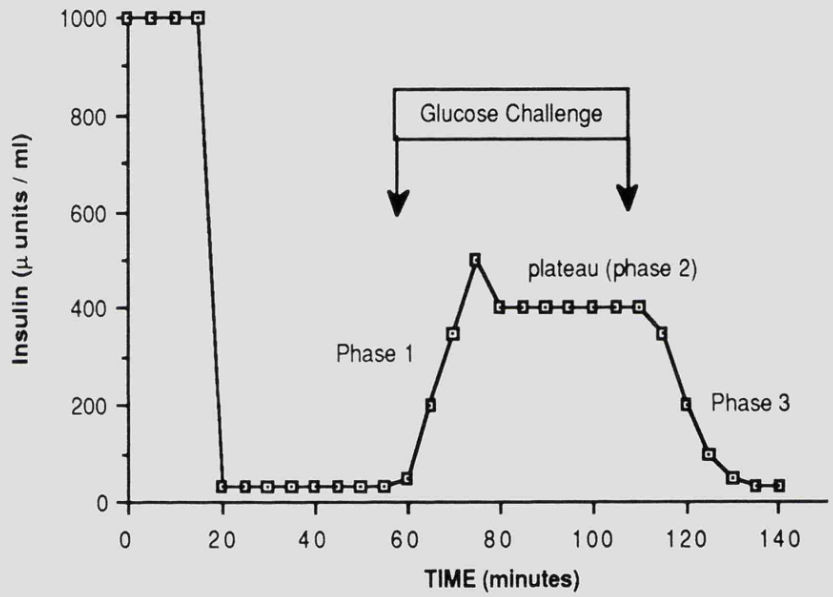

Fig. 9.2 Schematic graph of the expected insulin response of 'perfect' islets exposed to a glucose challenge. Briefly, as the islets are perifused with high glucose concentrations, they release stored insulin from their granules (Phase 1). After a peak of insulin release, there is a delay until newly synthesised insulin is released at a constant rate (plateau phase 2). When the glucose challenge is terminated, there is a corresponding fall off in insulin secreted (Phase 3). See text for full details.

FIG. 9.3

THE INSULIN SECRETION CURVE OF FRESHLY ISOLATED LDAF$_1$ PANCREATIC ISLETS IN RESPONSE TO A GLUCOSE CHALLENGE *(FIRST SERIES OF ISLETS)*

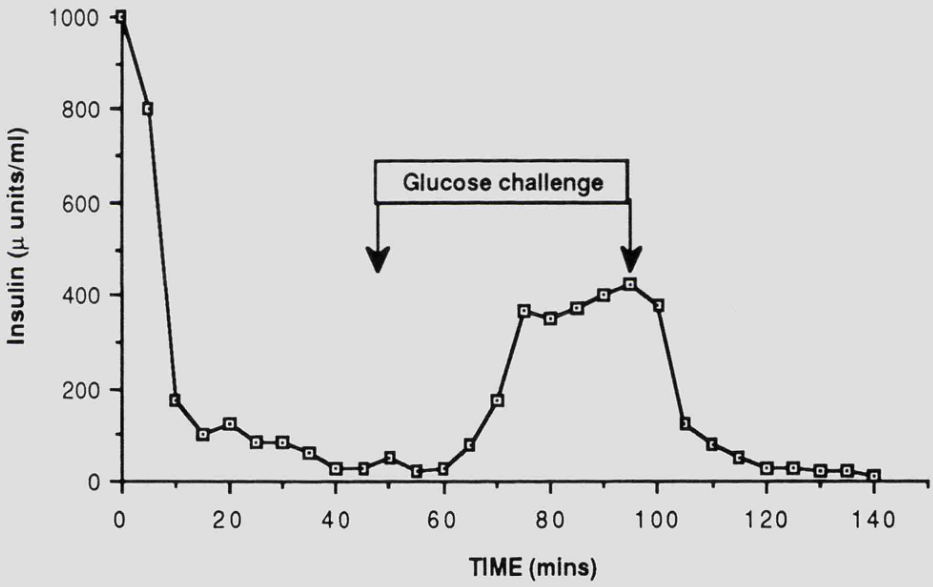

Fig 9.3 The insulin secretion curve of 150 freshly isolated LDAF$_1$ pancreatic islets in response to a glucose challenge during *in vitro* perifusion (first series of islets). It can be seen that, as expected, the response curve was almost identical to the schematic curve of Fig. 9.2. There was a sharp rise of insulin initially, followed by a plateau phase. When the glucose challenge was terminated the insulin secretion fell to background levels.

FIG. 9.4

THE INSULIN SECRETION CURVE OF FRESHLY ISOLATED LDAF$_1$ PANCREATIC ISLETS IN RESPONSE TO A GLUCOSE CHALLENGE. *(SECOND SERIES OF ISLETS)*

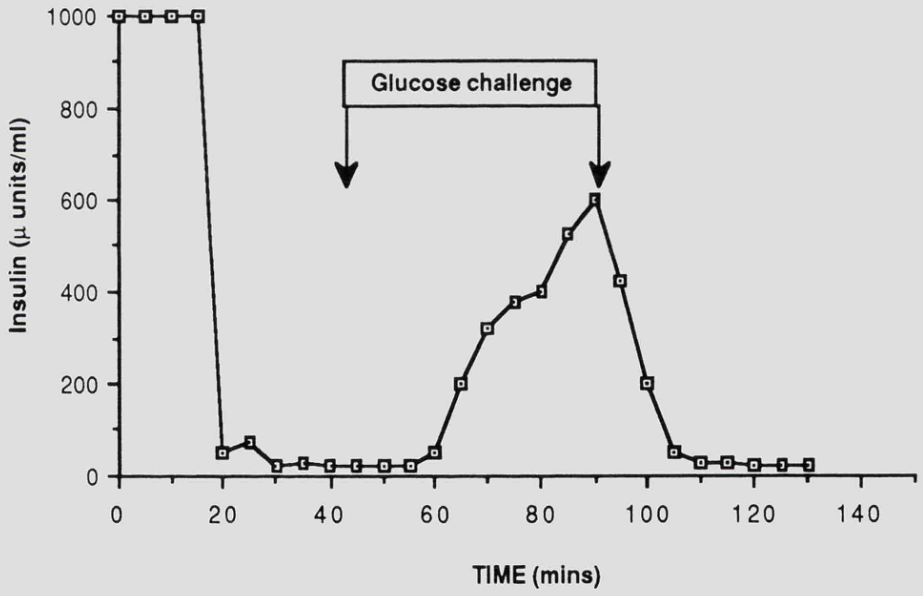

Fig. 9.4 The insulin secretion curve of 150 freshly isolated LDAF$_1$ pancreatic islets in response to a glucose challenge during *in vitro* perifusion (second series of islets). Once again there is a good response of insulin secretion to the glucose challenge. However, the plateau phase has been replaced by a second peak of high insulin release (see text for explanation). Finally there was an abrupt (normal) fall off of insulin secreted when the glucose challenge was removed.

TABLE 9.3

PREPARATION OF PERIFUSION SOLUTIONS

---

1) Standard Perifusion Solution Preparation

The following were dissolved in 1500 mls of Distilled Water :

NaCl	13446 mg
KCl	745 mg
$NaHCO_3$	4032 mg
$MgCl_2.6H_20$	407 mg
$CaCl_2$	555 mg
Albumin	200 mg

2) Low Glucose Perifusion Solution (60% Glucose)

60 mg D-Glucose were added to 100mls of the standard solution

3) High Glucose Perifusion Solution (300% Glucose)

300 mg D-Glucose were added to 100mls of the standard solution

---

Table 9.3 The preparation of the perifusion solutions. The standard preparation is made up in advance, and the morning of the perifusion determinations, the different glucose solutions are prepared.

FIG. 9.5

THE INSULIN SECRETION CURVE OF LDAF$_1$ PANCREATIC ISLETS, CULTURED FOR 24 HOURS, IN RESPONSE TO A GLUCOSE CHALLENGE.

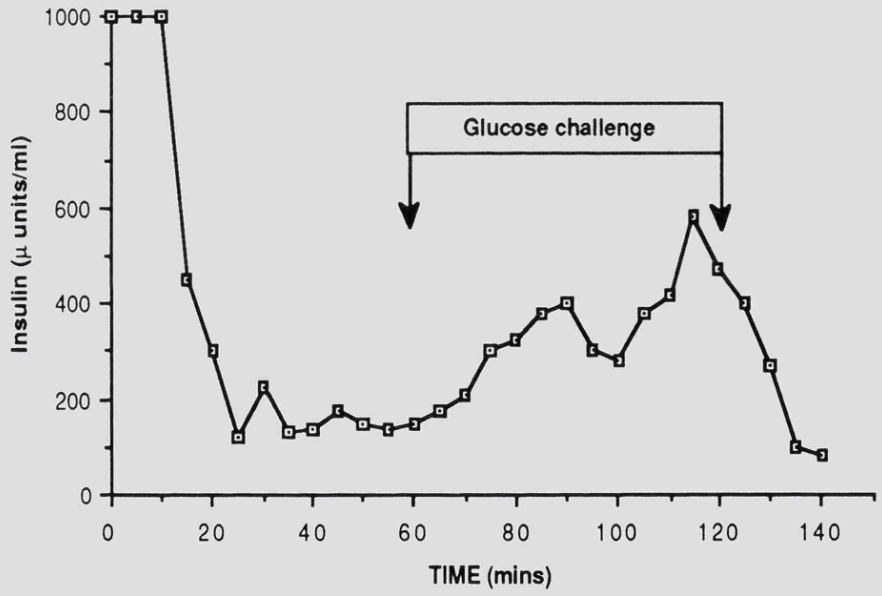

Fig. 9.5 The insulin secretion curve of 150 LDAF$_1$ islets, cultured at 37°C for 24 hours, in response to a glucose challenge by *in vitro* perifusion. This graph clearly demonstrates that the pancreatic islets were viable following prolonged culture. Once again the biphasic response of the secreted insulin is evident. Similarly, there is a normal reduction in insulin secretion when the glucose challenge is removed.

FIG. 9.6

THE INSULIN SECRETION CURVE OF LDAF$_1$ PANCREATIC ISLETS, ISOLATED FROM PANCREASES PERFUSED WITH ANTI-CLASS II MONOCLONAL ANTIBODIES.

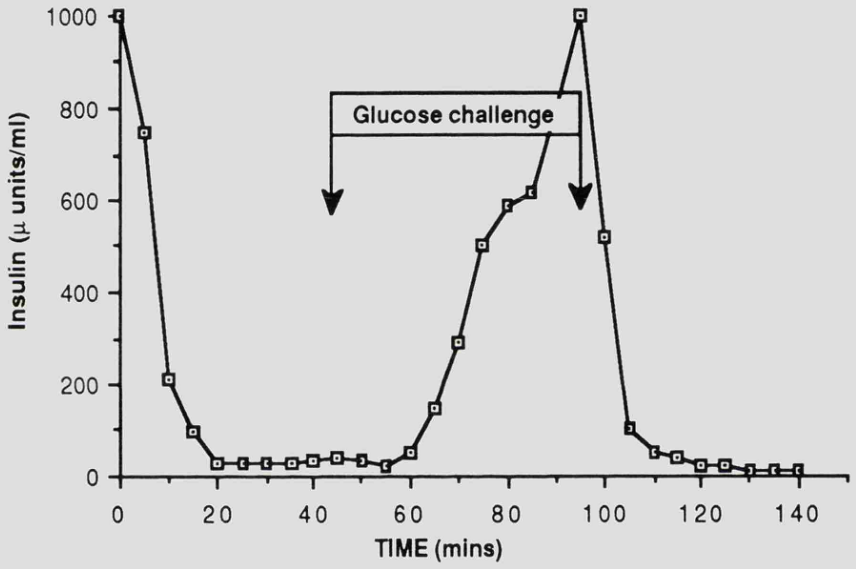

Fig. 9.6 The insulin response curve of 150 LDAF$_1$ islets isolated from pancreases perfused on the ex vivo perfusion circuit for three hours. It can be seen that there was an initial response of insulin secretion when the islets were first challenged with the glucose perifusion. However, there was an exaggerated response during the plateau phase. This may indicate some functional impairment to a few of the islets, but because the insulin secretion was abruptly stopped in response to the termination of the glucose challenge, this functional impairment was probably not significant.

9.4.2 Perifusion of Pancreatic Islets Cultured for 24 hours at 37°C

The *in vitro* response of 150 pancreatic islets cultured for 24 hours were studied in the perifusion circuit. The culture conditions employed were exactly the same as those described in 9.2.2 above.

The results of perifusion are shown in Fig 9.5. This graph shows that the response is essentially normal. There is a slight delay of phase 1 and its peak insulin level was subnormal indicating that the amount of stored insulin was lower. Nevertheless, the degranulation of the β cells in response to the glucose indicates normal function. The plateau phase (phase 2) has been replaced by another peak which indicates once again normal insulin synthesis. The lag of this phase was probably caused by a delay in protein synthesis secondary to suboptimal energy stores caused by the 24 hour culture. There was a normal 'cut-off' response of insulin output when the glucose challenge was removed (phase 3). Overall, therefore, there was no significant damage to the islets inflicted by the conditions of prolonged culture, as measured by *in vitro* perifusion.

9.4.3 The Insulin Response to Islets Isolated from Pancreases Perfused for Three Hours on the Ex Vivo Perfusion Circuit.

The fact that pancreases, perfused for three hours on the ex vivo perfusion circuit, reverse the experimentally induced diabetes following transplantation, indicates at least some, if not good preservation of the islet function within those organs (see Chapter 5.6). However, the assessment of the metabolic response by a whole pancreas upon transplantation to the recipient's hyperglycaemia is rather crude. i.e. the graft either controls the high blood sugar levels or it doesn't. Since only the immunological effects of ex vivo perfusion were to be studied, this 'crude' indicator of pancreatic viability was sufficient for the studies involved with allograft survival. However, a study of the functional effects of ex vivo perfusion on pancreas allografts is important if the perfusion

circuit was to be upgraded for studying larger animal models or even human pancreas perfusion. Thus, pancreases were perfused for 3 hours with complement, and the islets isolated from these organs following the ex vivo perfusion (see Chapter 5). The results of the perifusion are shown in Fig. 9.6.

It can be seen that this curve is somewhat abnormal. Phase 1 indicates that the islets released insulin in response to the glucose challenge, however, there was an abnormally high peak at the plateau phase, phase 2. This may represent further release of insulin from stored granules, but it may indicate some degree of damage, albeit slight, to the islets themselves. Some of the islets may have been irreversibly damaged and thus released all their insulin prior to cell destruction. This effect must be minimal, however, because there is a normal and abrupt cessation of insulin output at the termination of the glucose challenge.

9.5 DISCUSSION

The studies in this chapter confirm that isolated pancreatic islets, are amenable to *in vitro* immunoalteration by pretreatment with anti-Class II MAb's and complement. The mean allograft prolongation of 5.8 days when $LDAF_1$ pancreatic islets are treated with MAb and C', compared to controls, is similar to that of whole pancreatic allografts perfused for three hours with MAb and C', compared to their controls, a prolongation of 5.5 days. The rejection of the islets, however, occurs much earlier than whole pancreas allografts. Untreated islets ($LDAF_1$ to DA) reject between 5 and 8 days (mean 6.8 ± 1.0 days), and untreated whole pancreas allografts in the same strain combination reject between 9 and 12 days (mean 10.58 ± 0.9). The difference in these two means was highly significant ($p<0.001$). This confers with other studies which demonstrate that pancreatic islets are perhaps more immunogenic than intact whole pancreases and reject much earlier. However, the fact that the pancreatic islets were placed in the liver, and hence the portal circulation, may implicate an alternative explanation. The presentation of the alloantigens to the recipient may be of prime importance in the way that an alloaggressive response is elicited. Systemic drainage of the pancreatic grafts allows the donor antigens to be processed by lymph nodes and

spleen as well as processing in other sites including the liver. Portal vein injection of the islets, on the other hand, avoids the local uptake and presentation by lymph nodes. Perhaps it is this difference in site of 'antigen drainage' and subsequent processing and presentation, which may explain, in part, the differences in the rejection response of islets and whole grafts.

Second, the experiments in this chapter demonstrate quite clearly that the culture conditions used did not significantly alter pancreatic islet function as determined by perifusion. The *in vitro* response to a glucose challenge was similar to that of normal islets. Furthermore, the perifusion experiments verified that there was relatively good preservation of islet function in pancreases which had been perfused for three hours on the ex vivo circuit. The use of the *in vitro* viability assay as determined by a glucose challenge in the perifusion circuit is a useful indicator of pancreatic islet function, and could be applied to clinical studies.

SECTION III

Clinical Pancreas Transplantation and General Discussion

CHAPTER 10.

PANCREAS TRANSPLANTATION AT THE UNIVERSITY OF CHICAGO

10.1 INTRODUCTION

Organ transplantation has been the seminal achievement of clinical surgery in the second half of the twentieth century. The success in clinical transplantation has stimulated the development of immunology as a basic science, leading to advances applicable in the clinical arena not only back in transplantation, but also in virtually all other medical fields.

Kidney, liver, and heart transplantation remain lifesaving therapies for patients with end organ failure. In contrast, transplantation of the pancreas is employed for treatment of the disease process in diabetes mellitus, which is not eminently acutely life threatening. The complications of the disease process have already been discussed (Chapter 2). The necessity for a safe and reliable technique for the transplantation of the pancreas is balanced against the hope that the devastating progression of complications of the disease can be halted or even reversed.

With the recent advances in immunosuppression, particularly with the availability of the T cell specific immunosuppressant, cyclosporine, in 1983, the morbidity appeared to improve appreciably such that pancreas transplantation could be considered a reasonable therapeutic modality. In addition to providing a more specific immunosuppressive effect than that of drugs used previously, the use of cyclosporine has resulted in a decreased use of steroids leading to better healing and greater resisitance to overwhelming infection. With the improving results of pancreas transplantation in major centres, many of which are now reporting a one year pancreas graft survival rate between 70 and 86%, the Department of Transplant Surgery at the University of Chicago decided to embark on their clinical pancreas transplant programme in February 1987, utilising whole pancreas with exocrine drainage to the urinary bladder.

In conjuction with the research presented in this thesis, the clinical

pancreatic transplant programme was initiated with the intention that, should immune modulation be achieved by the pretreatment of pancreatic allografts in an animal model, then human organs, including kidneys and pancreases could be pretreated in a similar way. The donor organs would be flushed with anti-leucocyte common MAb's, prior to transplantation. There has already been some evidence that this technique is fairly successful in reducing the rejection response following transplantation.

Indeed, Brewer, Taube and colleagues, have flushed human renal allografts with a cocktail of two MAb's immediately before transplantation, with interesting results (Brewer *et al,* 1988). The antibodies used to pretreat 42 kidney allografts were rat anti-leucocyte common IgG_{2b} MAb's, YTH 24.5 and YTH 54.12; 10 other patients received allografts perfused with the mouse anti-leucocyte common IgG_{2a} MAb, F10-89-4. The renal grafts were biopsied following implantation, but before revascularisation, and immunohistochemical analysis performed to assess the adequacy of antibody labelling. Their results showed a good correlation between the reduction in severity and frequency of the allograft rejection episodes and the level of antibody binding. The more anti-leucocyte common MAb which was bound to the interstitial dendritic cells within the kidney, then less frequent and less severe were the allograft rejection episodes.

It is understood that after the initial 'learning curve' for clinical pancreatic transplantation at the University of Chicago, it would be conceivable that all allografts will be pretreated with anti-leucocyte common MAb's. These would include renal, pancreas and liver organs. However, before embarking on such a venture, not only was there a need for convincing evidence, in animals, that the pretreatment of allografts did infact reduce graft immunogenicity, but also the clinical pancreas transplant programme had to be well established and proven to be a safe therapeutic modality for Type I diabetes. The work in this thesis supports the first criterion, that the pretreatment of tissues with MAb's reduces graft immunogenicity. The second criterion, regarding the setting up of a clinically successful pancreas transplant programme will now be discussed.

10.2 EXPERIMENTAL TRANSPLANTATION IN PIGS

10.2.1 Rationale for Pancreas Transplantation in the Pig

Before the clinical programme was initiated, a series of experiments were performed in the pig for familiarisation with the surgical technique. As discussed in the first chapter, the technique deemed to be the most straight forward, associated with a lowest level of surgical complications, was whole pancreas transplantation including vascular anastomosis to the iliac vessels, with the pancreatic-duodenal conduit anastomosed to the recipient's bladder. Urinary drainage of the exocrine pancreas also had the added advantage that rejection episodes could be monitored by measuring a decline in urinary amylase levels. Furthermore, it allowed the chemistry laboratory to become familiar with a urinary amylase assay for assessing the large amounts of amylase expected to be secreted into the urine.

Total pancreactectomies were performed in 5 pairs of animals. The donor pig was sacrificed following removal of the pancreas, together with a duodenal segment. The proximal and distal parts of the duodenum were closed with a staple gun and a small incision made in the anti-mesenteric border. A Carrel patch was created on the coeliac artery of the donor pancreas and the portal vein isolated for venous drainage. The recipient pig underwent total pancreatectomy, with preservation of the delicate vascular arcade supplying the second and third parts of the duodenum. The pancreas was transplanted by anastomosing the vessels to the recipient's iliac artery and vein and the duodenal segment sutured to the bladder. In this way, the exocrine secretions from the pancreatic duct drained via the urinary tract. The blood sugars of the animals were monitored daily until pancreas rejection was manifested by a rise in serum blood glucose levels.

It must be emphasised that these transplants were only performed to familiarise the surgeons with the techniques, and also to assist the laboratory in developing an assay for urinary amylase. It was not intended as an immuno-

FIG. 10.1

GLUCOSE LEVELS AFTER PANCREATIC TRANSPLANTATION IN THE PIG

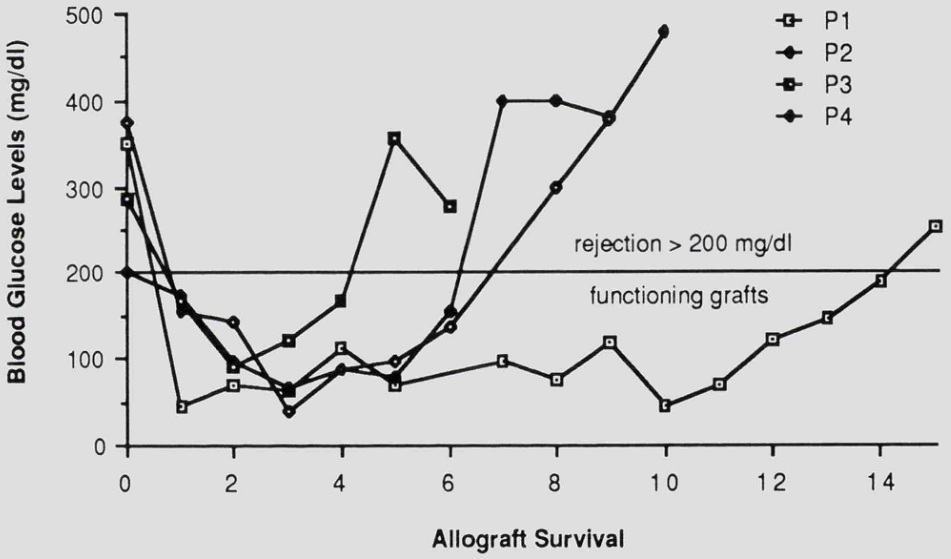

Fig. 10.1 This graph shows the glucose levels in 4 pigs (P1-4) following transplantation of pancreatic allografts. Rejection of the allograft was indicated by a rise in serum glucose levels above 200 mg/dl on two consecutive days.

In all recipients the blood sugars were controlled to normal levels post transplant. After this period of normoglycaemia, the pancreases were rejected on the following days: Pig 1 (P1) on day 15; pig 2 (P2) on day 7; pig 3 (P3) on day 5; pig 4 (P4) on day 8.

FIG. 10.2

URINARY AMYLASE LEVELS IN THE PIG FOLLOWING PANCREATIC TRANSPLANTATION WITH URINARY DIVERSION OF THE EXOCRINE SECRETIONS

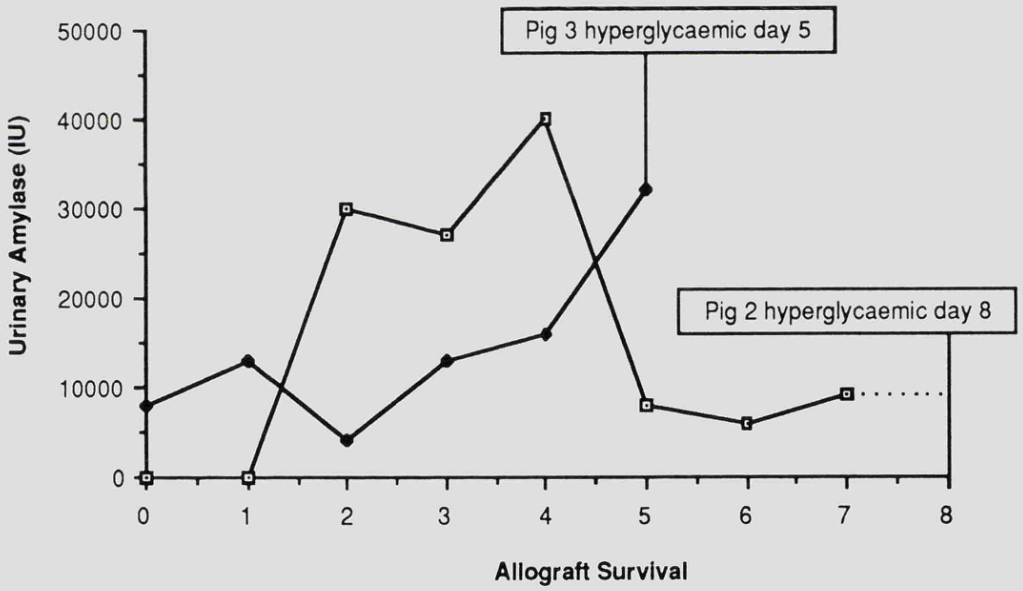

Fig. 10.2 This graph shows the urinary amylase levels of 2 pigs following pancreatic transplantation with exocrine diversion of the pancreatic secretions to the urinary bladder.

There was a fall in the urinary amylase levels in Pig 2 48 - 72 hours preceding a significant rise in blood sugar and clinically apparent allograft rejection. However, in Pig 3 there was no fall in the urinary amylase levels either before or coincident with late pancreatic allograft rejection

-logical study, and consequently no such tests were performed.

10.2.2 Results of the Pancreas Transplant in Pigs

Only 4 of the animals became normoglycaemic following this procedure. The fifth pancreas was damaged during the harvesting procedure and failed to function. All the remaining allografts functioned for between 5 and 15 days, mean allograft survival 8.8 ± 4.3 days. The daily serum glucose measurements are shown in Fig. 10.1. All 4 transplants therefore maintained euglycaemia following allografting until, in these untreated outbred recipients, rejection inevitably occurred. Autopsy on all the animals confirmed pancreas rejection.

The results of the urinary amylase levels are depicted in Fig. 10.2. Only the two animals in the series had serial urinary amylase levels measured with some accuracy. The amylase levels in the first transplant were undetermined or deemed inaccurate as the assay was established during this experiment. As mentioned, one pancreas did not function post transplant and there was no amylase determined in the urine. Of the two sets of urinary amylase levels obtained, one (pig No. 2) shows more or less the expected values. It can be seen that immediately following transplantation, coincident with a fall in blood sugar, the urinary amylase levels rose gradually as the pancreatic exocrine juices were secreted into the bladder. More importantly, it can be seen that 48 - 72 hours *before* a detectable rise in blood sugar, the level of urinary amylase fell precipitously. Thus, evidence of exocrine damage indicated by a fall in pancreatic amylase levels, preceded any clinical evidence of endocrine damage determined by a measurable rise in blood sugar.

In contrast, the levels of urinary amylase in pig No. 3 were not as one would expect. Following transplantation of the allograft there was a slow rise in the amylase levels and a peak coincident with hyperglycaemia on day 5. The amylase levels did not fall significantly, prior to allograft rejection. This remains somewhat inexplicable as autopsy confirmed severe rejection at this time. However, this was one of the first animals in which a full series of post operative urinary amylase levels were determined, and there may have been a dilutional

problem with the assay in the chemical laboratory, and abnormal readings perhaps obtained.

10.3 CLINICAL PANCREAS TRANSPLANTATION

10.3.1 Patient Selection - Recipient and Donor

All patients on the renal transplant waiting list who were Type I diabetic were referred for possible pancreas transplantation. Most of these patients were currently receiving haemodialysis, some were receiving peritoneal dialysis and a few in the predialysis period, though these patients were in end stage renal failure. Patients were considered suitable candidates for combined renal and pancreas transplantation on the basis of an extensive preoperative work up screening procedure. A list of some of the more important tests is given in Table 10.1.

Following a group meeting where the advantages and disadvantages were discussed with the patients, those selected for pancreas transplantation were subjected to blood tests as well as endocrine, cardiac, neurological, ophthalmic, gastro-intestinal and genito-urinary function tests. Most of these tests were to establish a base line for monitoring any change or improvement in the secondary complications of diabetes.

It would be pertinent to mention that the cardiac assessment was of paramount importance in evaluating the predictive risk of post operative myocardial infarction. Corry reported that 5 patients in his initial series died because of severe coronary artery disease (Corry and Ngheim, 1987). A tragic lesson that has benefitted subsequent patients. All patients in the series presented here had extensive cardiac function assessment. These tests included an ECG, echocardiogram, radionuclide angiography and occasionally a thallium scan. If any of these studies were abnormal, the patients would have coronary angiography, and possible referral for coronary artery bypass surgery prior to

TABLE 10.1

PREOPERATIVE WORKUP FOR RECIPIENTS OF PANCREATIC TRANSPLANTS

ENDOCRINE	GASTROINTESTINAL
Glucose	*Upper Endoscopy (if symptomatic)*
C-peptide levels	*Barium Enema (Age > 45 yrs)*
Insulin Assays	*Gall Bladder Ultrasound*
Insulin Antibody Titre	
Oral Glucose Tolerance Test	
Glucagon Stimulation Test	
Insulin Tolerance Test	
Hb A1c	
CARDIAC FUNCTION	**GENITO-URINARY**
12 lead ECG (+ stress)	*Urinalysis*
CXR (AP/Lat)	*Urine Cultures*
Echocardiogram	*Voiding Cystogram*
Radionuclide ventriculography (+ stress)	
? Thallium Scan	
? Angiography	
? Bypass Surgery	
OPHTHALMIC	**NEUROLOGICAL**
Visual Acuity and Fundoscopy	*Autonomic Nerve Function Tests*
Retinal Photography	*Skin Biopsy*
Retinal Fluoroscein Angiography	*Nerve Conduction Velocity Testing*
? Laser Treatment	*Galvanic Skin C-fibre response testing*

Table 10.1 A list of tests performed on potential recipients of pancreas transplantation.

transplantation if necessary.

Regarding donor selection, initially, patients between the ages of 16 and 50 were considered suitable for organ donation. They would have had to have suffered a major cerebral insult, and be clinically brain dead. Diabetes, chronic alcohol abuse, malignancy, chronic infections and a history of intravenous drug abuse were considered exclusion criteria. An acutely elevated plasma glucose was not considered an absolute exclusion criteria, and most of these patients were infact used for organ donation.

10.3.2 Operative Technique

A full description of the operative details is not required in this thesis, but some of the more important points will be addressed.

In the initial series, all patients underwent combined pancreas and renal transplantation, with both organs being placed in the groin. The pancreas was placed in the right iliac fossa with vascular anastomoses of the portal vein to the external iliac vein and the coeliac axis to the external or common iliac artery. The whole pancreas was used with a duodenal conduit anastomosed to the bladder (Plate 10.1). The spleen was left attached to the pancreas throughout the procedure and only removed just prior to skin closure; it was thought that this helped to prevent graft thrombosis (Plate 10.2). In all cases, following flushing with Eurocollins solution and subsequently with silica gel filtered plasma, the pancreas was transplanted within 6 hours of the donor pancreatectomy, and was immediately followed by transplantation of a kidney from the same donor into the contralateral iliac fossa. Insulin therapy, in the form of a continuous intravenous infusion of both insulin and glucose, was continued postoperatively for 1-4 days until patients began to eat. For all successful transplants, when intravenous infusions of insulin were stopped the patients remained normoglycaemic. Anticoagulant therapy consisted of subcutaneous heparin and intravenous dextran (Reomacrodex) until the patients were mobile. The patients continued to take aspirin 300 mg and dipyridimole, 500 mg, daily. Isotope perfusion scans were performed on the first post operative day in all patients, and most underwent cystoscopic examination of the duodenal conduit at three months post transplant

PLATE 10.1

SCHEMATIC DIAGRAM DEMONSTRATING THE SURGICAL TECHNIQUE OF PANCREAS TRANSPLANTATION

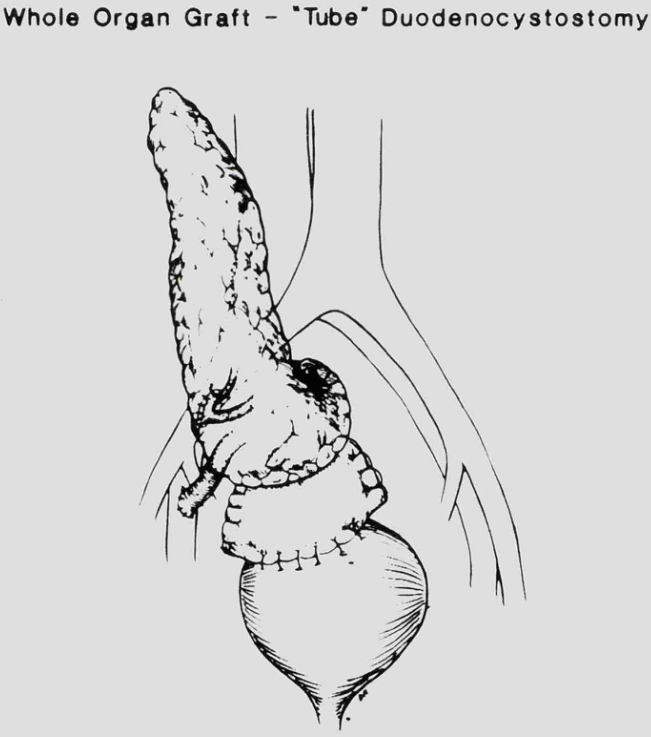

Plate 10.1 This schematic diagram demonstrates the bladder drainage technique used for transplantation of the pancreas. A duodenal conduit was fashioned and anastomosed to the recipient's bladder. This "tube" duodenocystostomy allowed pancreatic exocrine secretions to be excreted with the urine.

PLATE 10.2

PHOTOGRAPH OF THE PANCREAS AND SPLEEN PRIOR TO TRANSPLANTATION

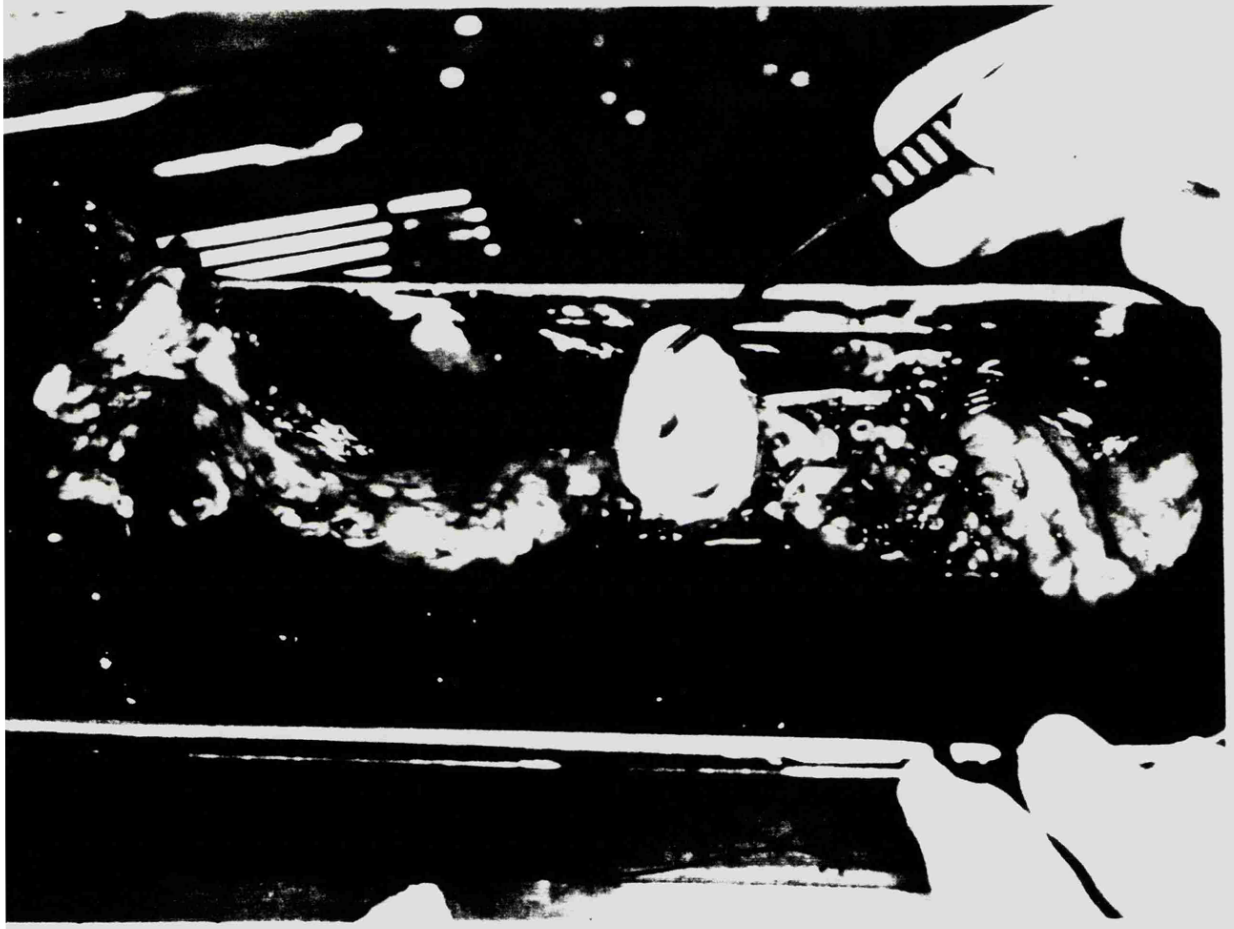

Plate 10.2 This is a photograph of an isolated pancreas prepared for transplantation. The Carrel patch surrounding the coeliac and superior mesenteric arteries is demonstrated. The open ends of the duodenal segment (right) have been stapled and oversewn with silk sutures. The spleen (left) is left attached to the pancreas, but removed at the end of the procedure following revascularisation of the pancreas and creation of the duodeno-cystostomy.

PLATE 10.3

PHOTOGRAPH OF INFARCTED PANCREAS ALLOGRAFT DUE TO GRAFT PORTAL VEIN THROMBOSIS

Plate 10.3 This is a photograph of a pancreas graft removed the day after transplantation because of non-function. It can be seen that there is extensive thrombus in the portal vein.

PLATE 10.4

PHOTOGRAPH OF PERFUSION SCANS ON DAY 1 POST TRANSPLANT

Plate 10.4 These are photographs of perfusion scans performed one day following combined kidney and pancreas allografting. The scan on the left is normal showing uptake in both the kidney (right groin) and pancreas (left groin).

The scan on the right is abnormal. Despite excellent uptake in the renal allograft (arrowed), there is no uptake in the pancreas. This patient developed portal vein thrombosis in the pancreatic graft, which became infarcted. It was safely removed on the first post operative day; the kidney continues to function normally.

PLATE 10.5

PHOTOGRAPH OF THE DUODENAL CONDUIT TAKEN FROM INSIDE THE BLADDER

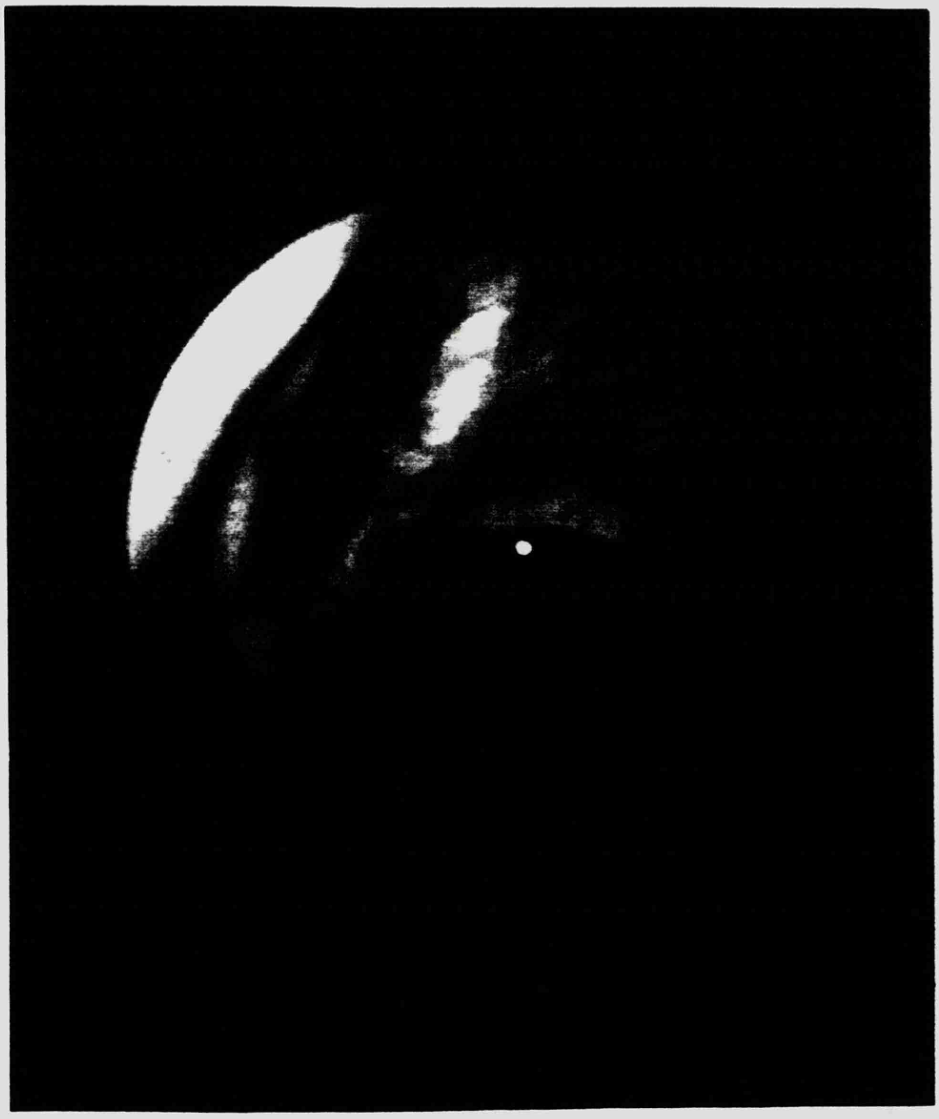

Plate 10.5 This is a photograph of a cystoscopic view taken from within the bladder, of the duodenal conduit three months following pancreas transplantation. The air bubble at the top of the bladder shown. It can be seen that the duodenal mucosa is healthy. Biopsies of both pancreas and duodenal mucosa can be performed relatively safely via the cystoscope.

PLATE 10.6

PHOTOGRAPH OF A CYSTOGRAM IN A PATIENT FOLLOWING PANCREAS TRANSPLANTATION

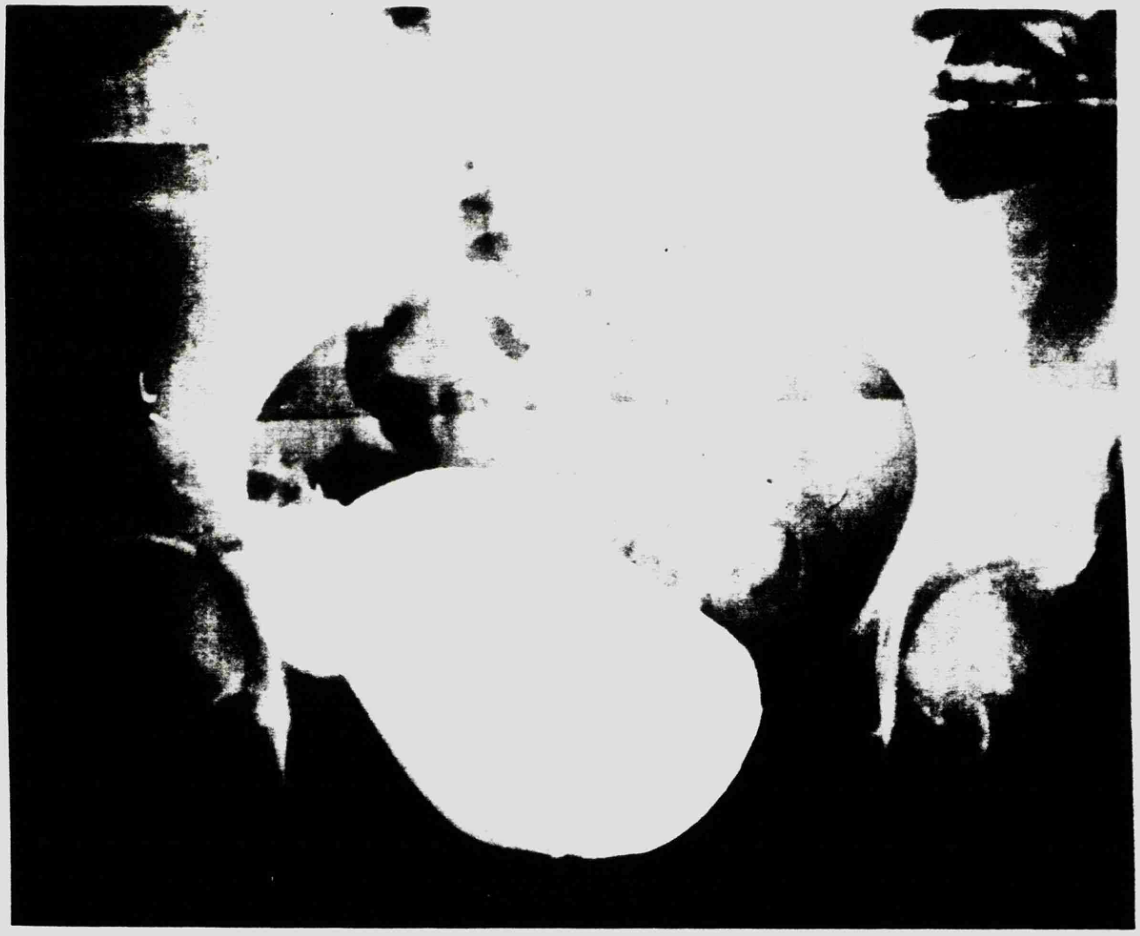

Plate 10.6 This is a photograph of a cystogram in a patient three months after pancreas transplantation. The bladder is outlined with contrast and the transplanted duodenum appears as a bladder diverticulum on the right. Note there is filling of the pancreatic duct (arrowed) verifying duct patency, and note the absence of ureteric reflux on the contralateral side.

FIG. 10.3

ONE YEAR PATIENT SURVIVAL, RENAL ALLOGRAFT SURVIVAL AND PANCREAS ALLOGRAFT SURVIVAL FOLLOWING COMBINED PANCREAS/KIDNEY TRANSPLANT

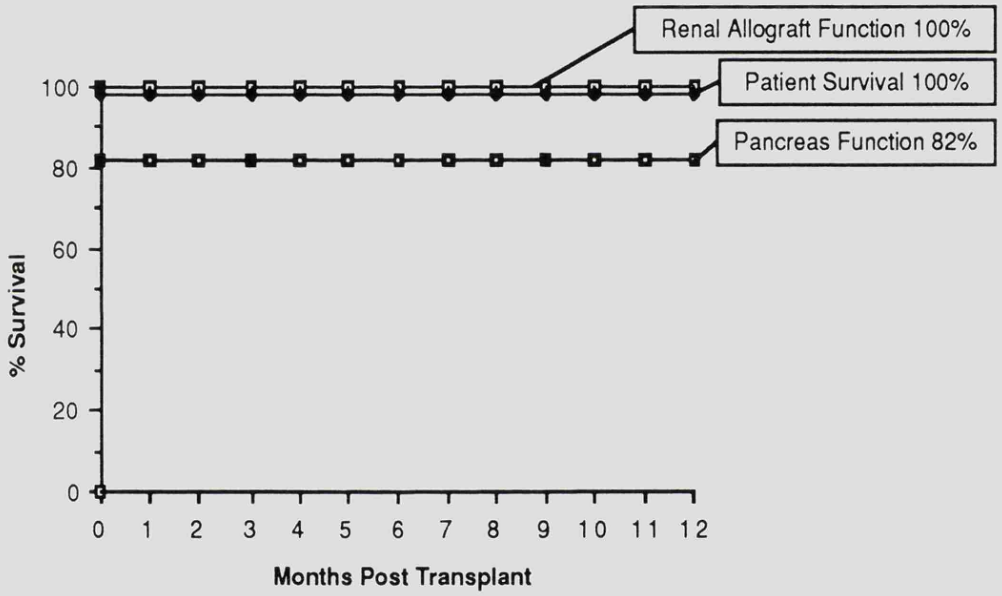

Fig. 10.3 Graph showing the actuarial survival of patients receiving combined pancreas and renal allografts at one year post transplant (n = 11). This graph also shows the function of both the kidney and pancreas allograft at one year. It can be seen that with a mean follow up of 15.4 ± 4.6 months (range 9 - 21 months), patient graft survival was 100%, renal allograft survaival 100% and pancreas allograft survival 82%.

TABLE 10.2

GRAFT FUNCTION OF COMBINED PANCREAS AND KIDNEY ALLOGRAFTS IN RECIPIENTS

Patient	Fasting Plasma Glucose (mg/dl)	BUN (mg/dl)	Creatinine (mg/dl)
1. SA	117	27	1.6
2. LC	88	30	1.4
3. DW	226[a]	31	1.6
4. PD	99	27	2.2
5. PH	94	9	1.4
6. KG	74	27	1.0
7. AB	170[b]	29	1.1
8. JM	88	29	1.6
9. BY	72	30	1.2
10.PE	101	40	2.3
11.JR	93	22	1.5

Table 10.2 Table showing the current (February 1988) renal and pancreas function for combined renal and pancreas allografts in 11 consecutive patients. 9 of the 11 patients had functioning pancreatic allografts and all patients had good renal function.

[a] Graft pancreatectomy at 24 hours post transplant for venous thrombosis. Patient resumed insulin therapy.

[b] Graft pancreatectomy on day 4 post transplant for graft pancreatitis. Patient resumed insulin therapy.

(Plate 10.5). At this time duodenal biopsies would allow histological assessment of the duodenal mucosa.

10.3.3 Results

In this initial series, a total of eleven combined pancreas and kidney transplants were performed. Nine of the pancreases successfully controlled the hyperglycaemia and in these patients insulin therapy was discontinued representing an 82% successful graft function rate (mean follow up survival 15.4 ± 4.6 months). In two patients, the pancreases failed to function postoperatively. In the first patient, the third in the series, the pancreas graft developed portal vein thrombosis (Plate 10.3), probably within minutes of the procedure. There was no significant level of amylase detected in the urine postoperatively, and a radio-labelled isotope perfusion scan confirmed complete loss of vascular supply to the organ (Plate10.4). The pancreas was removed on the first post operative day; the kidney continued to function normally.

The second pancreas transplant failure occurred in the seventh patient in the series. This pancreas developed post operative graft pancreatitis and was removed safely on the third day following transplantation. Once again the renal function remained normal and was unaffected by the pancreatitis or graft pancreactectomy. Seven of the nine successful combined pancreas/kidney transplant recipients (78%) experienced an episode of allograft rejection despite induction immunosuppression with anti-thymocyte globulin and maintenence therapy with cyclosporine, axothiaprine and prednisone. This rejection rate is comparable to the 60% incidence of ejection observed in patients receiving kidney allografts alone at the University of Chicago (Thistlethwaite *et al,* 1986). In all cases of rejection in the combined pancreas/kidney transplants recipients, rejection first clinically presented with an increase in serum creatinine and was confirmed by renal biopsy. There was relatively little clinical evidence of rejection in the pancreas, although no biopsies were performed for histological examination. Serum glucose remained normal in all patients until bolus steroid therapy was initiated at the time of rejection episodes. Excreted urinary amylase was found to be decreased significantly in only 3 cases at the time of initial diagnosis of

FIG. 10.4

GLUCOSE TOLERANCE CURVE IN A PATIENT SIX MONTHS AFTER PANCREAS TRANSPLANTATION

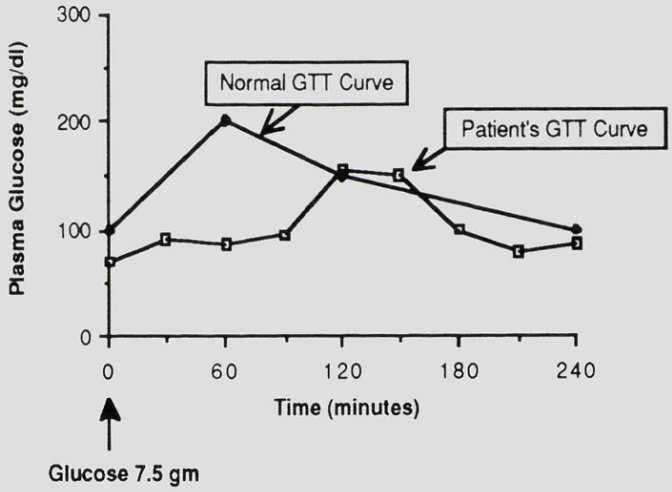

Fig. 10.4 This graph shows the blood glucose levels in a patient 6 months after pancreas transplantation, during an oral glucose tolerance test (GTT), in response to an oral glucose load of 7.5 g.

It can be seen that although the rise in blood sugar was delayed, as compared to non-diabetic controls, the plasma glucose levels of the patient remained within normal parameters.

FIG. 10.5 and FIG. 10.6

SERUM C-PEPTIDE LEVELS AND PLASMA INSULIN LEVELS AFTER PANCREAS TRANSPLANTATION IN RESPONSE TO AN ORAL GLUCOSE LOAD

FIG. 10.5

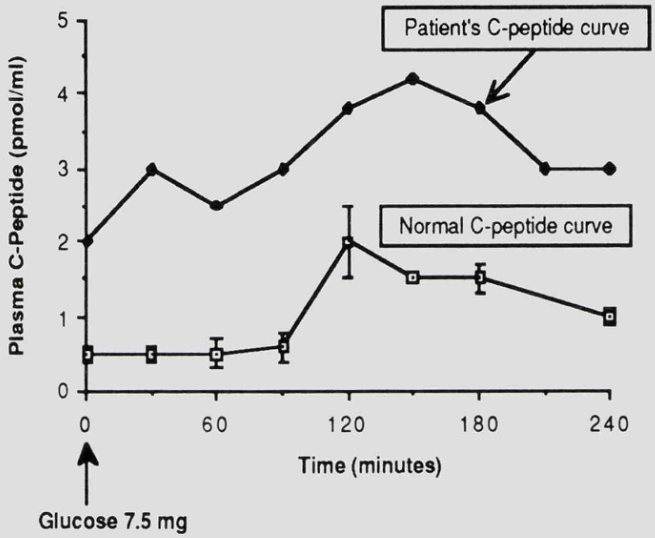

FIG. 10.6

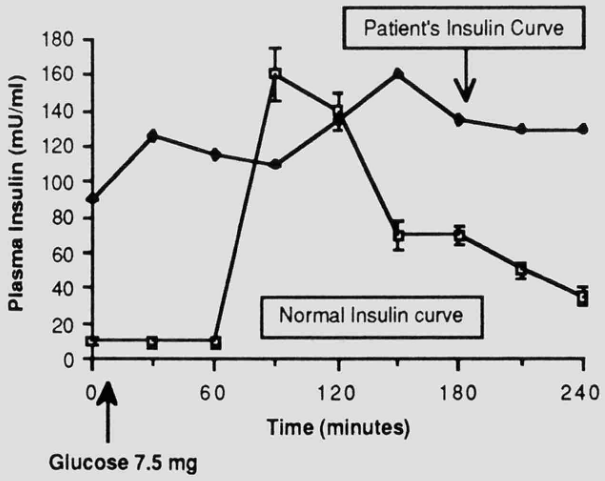

Fig. 10.5 (above) and Fig. 10.6. These graphs shows the serum C-peptide levels (above) and plasma insulin levels (below) in response to an oral glucose load of 7.5 g, in a patient 6 months after pancreas transplantation.

TABLE 10.3

SYMPATHETIC SKIN (C - FIBRE) RESPONSE IN PANCREAS TRANSPLANT RECIPIENTS

PATIENT	FOOT		HAND	
	Pre Tx	6 mth Post Tx	Pre Tx	6 mth Post Tx
1.	+ve or ↓	+ve	+ve	+ve
2.	- ve	+ve	+ve	+ve
4.	- ve	+ve	+ve	+ve
5.	- ve	-ve	-ve	-ve

+ve Normal Response

-ve Absent Response

↓ Present Response, but decreased.

Table 10.3 This table compares the sympathetic skin (C - fibre) response pretransplant and 6 months post pancreatic transplant in the first four patients with successful pancreas transplants.

It demonstrates an improvement in the sympathetic response in the foot in patients 1, 2 and 4. Patient No. 3 is excluded from this study because of a failed pancreas graft (see text).

rejection. All rejection episodes not responsive to pulses of steroids were successfully treated with the anti-T cell receptor monoclonal antibody, OKT3.

Fig. 10.4 shows a typical oral glucose tolerance test performed in one of the patients 6 months after pancreas/kidney transplantation. Although there was a slight delay in the patient's return to normoglycaemia following glucose ingestion, the peak plasma glucose level is not elevated. Fig. 10.5 shows the plasma C-peptide response following an oral glucose load. It demonstrates abnormally high C-peptide levels because of the systemic drainage of the insulin from the pancreatic graft into the inferior vena cava of the recipient. A similar abnormal curve is seen in the response of insulin to a glucose load (Fig. 10.6).

Preliminary neurological data suggests some improvement of sudomotor and sympathetic nerve function. It is well established that diabetics tend to loose their ability to sweat, particularly in the late stages of the disease process when peripheral neuropathy is evident. Early on in the disease, some diabetics have a tendency to hyper-respond to stimuli and have an increased sudomotor response. Six months following pancreas transplantation, in those patients studied (n = 4), all of them had documented improvement of the sweat response. Further improvement of the diabetic neuropathy following pancreas transplantation is evident when the results of the sympathetic skin (C-fibre) response were analysed. Of the first four patients with a successfully functioning graft at six months post transplant, three patients had objective improvement of sympathetic skin response (Table 10.3).

In this context, it must be reiterated that all these patients had combined renal-pancreas transplants and it is well documented that neuropathy improves following a single renal allograft.

10.3.4 Complications

General surgical complications were few. There were no abscesses, haematomas or pancreatic fistulae associated with the pancreatic transplants. As mentioned, two pancreases were removed post operatively, and those patients have resumed insulin therapy. Despite anticoagulation therapy one patient developed a deep vein thrombosis and a sub-clinical pulmonary embolus, detected on a V-Q scan. Two patients developed oral candidiasis, and one had fairly severe

cyclosporine toxicity, all of which were resolved by a reduction in immunosuppression.

10.4 DISCUSSION

The 82% allograft survival rate from the first year of the University of Chicago Pancreas Transplant Programme attests to the fact that pancreas transplantation can be performed as successfully as transplantation of other organs. With 100% patient survival achieved, it is now appropriate to expand the arena of transplantation from exclusively emergency life-saving procedures to also include elective treatment of a devastating, but not eminently life-threatening disorder, Type I diabetes. Challenges still remain to improve pancreas transplant success rates. Paramount amoung these is the development of a method to detect rejection early in the course of this process before extensive damage to the pancreas has occurred. Until a reliable method of detecting pancreas allograft rejection has been established, combined pancreas/kidney transplant, whereby rejection can be monitored in the kidney, will be the only form of pancreas transplantation to achieve a high level of graft survival. But, in a sense, limiting pancreas transplantation to diabetics with uraemia and, thus, with other well advanced secondary complications, defeats the purpose of the transplant itself i.e. to prevent the development of these complications.

Although the data presented here confirms improvement in the neuropathies, one very important point must be emphasised. These patients received combined kidney and pancreas transplants, and the improvement in the neurological status cannot be attributed to the pancreas alone. Uraemia is known to cause a neuropathy and the improved results may well be due to the effect of renal transplantation and concomitant improvement of renal function. Until a controlled trial between pancreas transplantation alone versus no transplantation at all, performed in pre-uraemic patients not requiring renal allografting is performed, the isolated effects (and benefits) of pancreas transplantation will remain anecdotal.

Many centres, including the University of Chicago, are reporting one year

allograft survival figures above 70% utilising whole pancreas transplantation with a duodenal conduit for exocrine drainage into the bladder. These include the group in Wisconsin (86%), in Iowa (70%), and in Minnesota (72%). This is due to the choice of technique and better immunosuppression. Segmental pancreatic allografting is becoming safer and is strongly advocated in Europe, and the results reported in the Pancreas Transplant Registry, do not reflect any difference in the overall world series between segmental and whole pancreas transplantation, although there are not many individual centres reporting a one year graft survival greater than 70% utilising a segmental approach.

That successful pancreas transplantion is now a relatively safe and reliable procedure in Chicago, the second half of the programme will commence soon. Donor pancreases and kidneys will be flushed with human anti-leucocyte common MAb's, and the effect on reducing the frequency and severity of rejection episodes following allografting will be determined. Providing this procedure is a safe one and confers no risk to the transplant recipient, then even if the benefits are small, they would be greatly welcomed.

CHAPTER 11

GENERAL DISCUSSION AND DISCUSSION OF THESIS

11.1 INTRODUCTION

Manipulation of an organ allograft to reduce its immunogenicity is a potential alternative in transplantation which could lower the incidence of rejection without any risk to the transplant recipient. Most attempts at immunoalteration in the past have involved donor pretreatment regimens which are only optimal 2 to 5 days prior to organ procurement; such regimens are not clinically feasible. Recently, it has been demonstrated that depletion of passenger leucocytes from isolated murine pancreatic islets could be accomplished by in vitro treatment with anti-dendritic cell monoclonal antibody and complement, and that treated pancreatic islets survived indefinitely when transplanted to recipients which normally reject untreated islets (Faustman *et al,* 1984). There are several other *in vitro* techniques available to either individual cells or groups of cells, which permit immunoalteration by some form of pretreatment protocol. The basis of this thesis was to establish whether immune manipulation by pretreatment with anti-Class II monoclonal antibodies could be applied to whole organs.

To achieve this aim, the hypothesis that pretreatment with anti-Class II MAb's could reduce the immunogenicity of transplantable tissue had to be confirmed in the animal model used and secondly, a whole organ transplant model had to be developed, in such a way that, if a reduction in graft immunogenicity was achieved, adaptation for clinical use could, theoretically, be established.

The thesis, therefore, comprises two complimentary groups of experiments. The first, in Section II, investigates the possible efficacy of MAb pretreatment of both whole pancreatic grafts and isolated pancreatic islets. The experiments confirm that pretreatment with anti-Class II monoclonal antibodies does decrease graft immunogenicity. The *ex vivo* perfusion studies demonstrate

that the pretreatment of intact whole pancreases with anti-Class II MAb's results in a significant prolongation of allograft survival compared to untreated controls. Also, the *in vitro* studies of pancreatic islets in a mixed islet lymphocyte culture, demonstrate that pretreatment with anti-Class II MAb's abrogates their capacity to stimulate the proliferation of allogeneic T cells.

The second group of studies, as discussed in Section III, establishes that clinical pancreatic transplantation is a safe and reliable surgical procedure.

11.2 ANTIGEN PROCESSING AND ANTIGEN PRESENTING CELLS

Allograft rejection can be divided into two phases. The first would include the induction of the immune response, involving grafting of the allogeneic tissues with concomitant release or exposure of antigenic components, and processing and recognition of the antigens by the host with stimulation of the immune system toward the relevant antigens. The second would include the effector limb of the rejection response and involve the destruction of the graft itself.

The results presented in this thesis confirm the central role played by the antigen presenting cell in the generation of the T cell immune response. This has been extensively investigated by others, yet neither the pathway of antigen through the cell nor the precise biochemical events in the process has been elucidated (Unanue, 1980). Although a number of studies have been devoted to this problem, it has so far been impossible to separate adequately the biochemical events connected with antigen presentation from the general catabolic processes of the cell (Allen *et al,* 1984; Chesnut, Colon and Grey, 1982).

A better appreciation of the essential nature of antigen presentation was due, in part, to the discovery of the restriction imposed on cellular interactions by products of the major histocompatibility gene complex (MHC) (Rosenthal and Schevach, 1973). The existence of Class I and Class II antigens determined by MHC genes had been recognised for some time (Bach *et al,* 1976). Class II antigens, or antigens presented in combination with Class II, induce T-helper (Th) cells (Miller *et al,* 1976). These cells mediate delayed type hypersensitivity

responses against cells bearing the Class II antigen which was presented on the original stimulator cell. Class I antigens, on the other hand, or antigens presented in association with Class I antigens, induce cytotoxic T-cells (Tc), which mediate their response only against cells bearing the Class I antigens represented on the original antigen-presenting cell (Doherty *et al,* 1976).

The expression of abundant quantities of Ia antigen on APC's is not disputed, and though the regulation of the mechanisms involved in antigen presentation are complex, they have been reviewed by several authors (Unanue *et al,* 1984; Marrack, 1987; Chain *et al,* 1986).

The expression of Ia antigens on the APC may well be a crucial step in the control of immune responsiveness. The extent of Ia expression can be regulated in several ways. The interaction of Ia positive macrophages with some agents that trigger phagocytosis will maintain Ia expression and retard the transition from Ia positivity to Ia negativity (Beller and Unanue, 1981). Also, the products of antigen-stimulated T cells preferentially induce Ia expression in previously immature Ia negative macrophages (Scher *et al,* 1982), and certain inhibitory molecules diminish the expression of Ia antigens (Snyder *et al,* 1982). The various Ia positive, antigen presenting cells interact to a varying degree with different antigens and may cooperate with each other at different stages in the immune process to ensure effective triggering of the T helper cell. It is likely that because of the ubiquitous representation of Ia positive macrophages, and their extensive capacity to take up and handle foreign antigens, the interaction plays a critical role in the generation of T helper cells reactive not only to microbes but also foreign proteins circulating in blood or extravascular fluids. Ia presentation, therefore, is of fundamental importance in the alloaggressive response.

It is the interaction between the Class II MHC products and the T helper cell which is the crucial element in the initiation of this alloaggession, and it is the activation of the T helper cells by the presenting cells, expressing these Ia antigens, which induces the immune response. This activation does not appear to be induced by free antigen but only by antigen presented by an accessory cell that displays the Class II glycoproteins of the MHC, i.e. the Ia antigens, (Benacerraf, 1981). The Class II antigen presenting cell serves to elicit the initiation of the immune response in organ allografts, but actual knowledge of the sequence of

events eludes immunologists at present.

In vitro studies have helped clarify some of the effects (Fig. 11.1). The T helper cells are stimulated into cell division by the Ia^+ dendritic cell (DC) in the allogeneic mixed lymphocyte reaction (MLR) (Schwartz *et al,* 1978). The T helper cells recognise Class II antigens predominantly and produce interleukin 2 (IL-2). These cells require the DC as a specialised antigen presenting cell, in contrast to cytotoxic T cell precursors, which are driven to effector function by any type of allogeneic stimulator cell in combination with IL-2 (Golding and Singer, 1984).

The results of the MLC's presented in this thesis, between $LDAF_1$ stimulator T cells and DA responder T cells, with the addition of either $LDAF_1$ or DA APC's, confirm that T cell proliferation is dependent on Class II presentation and the presence of the accessory cells. One to two days after the initiation of the MLC, small clusters were seen in culture, presumably made up of T blasts and DCs. These clusters also arise during a syngeneic MLC and in the primary antibody responses *in vitro,* but were not significant in the allogeneic T cell cultures between the particular strain combination used. These clusters were important functionally since the clustered T cells produced IL-2, whereas the non-clustered cells probably did not. Consequently, it was the clustered T cells that proliferated.

The events occuring in the allograft rejection response are thought to be analogous to the *in vitro* mechanisms. Immune induction is dependent upon graft antigen, presented upon either donor or host APC coming into contact with the relevant allo-reactive cells (ARC). This may occur by migration of ARC through the graft, or by the migration to, or the presence of, APC in the regional lymph node or spleen of the host (Pedersen and Morris, 1970; Tilney and Ford, 1974). In the course of the orderly procession of the recirculating pool of lymphocytes through these organs the ARC may make contact with the foreign Class II antigen. At these sites, following appropriate presentation of antigens, interactions between subsets of cells including both circulating and sessile can occur. Once specific effector cells enter the graft, there is a second phase of proliferation and clonal expansion (Ascher *et al,* 1981). The *in situ* expansion of

the response is associated with the release of various lymphokines and inflammatory mediators, which increase vascular permeability and attract leucocytes, including lymphocytes, from the circulation into the graft (Hopt *et al,* 1983).

It can be concluded that the Ia antigens, encoded for in the Ir gene locus, play an essential role in antigen recognition by T lymphocytes involved in helper functions (Swierkosz *et al,* 1978; Hodes *et al,* 1980), proliferation (Schwartz *et al,* 1978; Thomas *et al,* 1977), and delayed-type hypersensitivity (Miller *et al,* 1976). The recognition by such T cells of the polymorphism of allogeneic Ia itself, or self Ia molecules in association with nominal antigen on an Ia-bearing accessory cell is essential for T cell activation and results in the MHC Class II restriction of responses. That T helper cells are of paramount importance in the initiation and continuation of graft rejection is not disputed. Yet the molecular and cellular events that lead to the actual activation of the allogeneic T cell response remain controversial. The antigen recognition and immune induction were considered to be part of the same process, but there is increasing interest in which cell 'presents' the foreign antigen to the T helper cell. The findings in this thesis would corroborate that the Class II MHC antigen positive cells within the allograft play an important role in this field. Both the *ex vivo* perfusion studies and the *in vitro* lymphocyte culture experiments verify that the immunogenicity of the pancreatic tissue is significantly reduced when pretreated with anti-Class MAb's. The presentation of the foreign antigen is suboptimal.

The evidence presented here provides further proof that the expression and presentation of Class II MHC antigens are crucial for optimal alloaggression. In the mixed lymphocyte studies, there was no proliferation of T cells when two allogeneic groups of T cells were co-cultured. It was only the addition of Class II positive cells to these cultures that resulted in T cell proliferation. Furthermore, pretreatment of pancreatic islets with anti-Class II MAb and complement abolished the in vitro allogeneic proliferative response of T cells. Whether this effect was due to depletion of the Ia +ve cells or just inactivation of them is uncertain. Certainly, similar effects can be achieved when islets are pretreated with antibody alone, and no complement. This would eliminate the possibility of complement-mediated lysis of the dendritic cells and raise the possibility that the

immune modulation is caused by a 'blocking effect' of the Class II antigens to allopresentation.

Support for the blocking effect is evident in the ex vivo perfusion experiments. Even after 3 hours of continual perfusion with excess MAb and rabbit complement, at 37°C, Class II antigen positive cells could still be identified in the pancreases. Immunohistochemical labelling of sections of these pancreases by anti-Class II probes, clearly demonstrated the presence of these cells following perfusion. It may have been that the cells were dead and that because no phagocytic cells were present in the perfusate, because anti-Class II MAb's and C' were used together with buffy coat depleted blood, an efficient mechanism for the removal of these cells was missing.

If the cells were killed, theoretically, they could still have Class II glycoproteins on their cell surface reactive with the immunoprobes used in these studies. Attempts to identify the supposedly killed cells, individually, proved futile. Electron microscopic evaluation was abandoned because the Ia +ve cells could not be distinguished from surrounding panceatic acinar or islet cells. Also, the addition of Trypan Blue to the perfusate, in an attempt to identify the dead dendritic cells by their inability to exclude the dye and therefore stain blue, was similarly unsuccessful. Multiple microscopic crystals of trypan blue within the interstitium of the pancreatic tissue, interfered with the assessment of the numbers of 'blue' cells and the results were inconclusive.

To explain the immunological benefit of perfusing organ allografts with anti-Class II MAb's, evident from the transplantation studies, one can only conclude that the Ia +ve dendritic cells were *inactivated*, though actual killing has not been excluded. In none of the transplant experiments was indefinite allograft survival achieved. The double labelling experiments confirmed that all the Class II positive cells in the pancreases were labelled with anti-Class II MAb during ex vivo perfusion, and yet all were rejected, though most in a significantly delayed fashion. If these cells were not killed or damaged by the binding of the complement fixing MAb, in the presence of C', then recovery of these cells and their active participation in the presentation of their Class II antigens would be one alternative to explain this phenomenon. However, it is more likely that the delayed rejection of these perfused allografts was due to permanent functional ability of the Class II positive cells to present antigen. The experiments involving

FIG. 11.1

SCHEMATIC DIAGRAM OF THE INITIAL EVENTS OCCURRING IN ALLOGRAFT REJECTION.

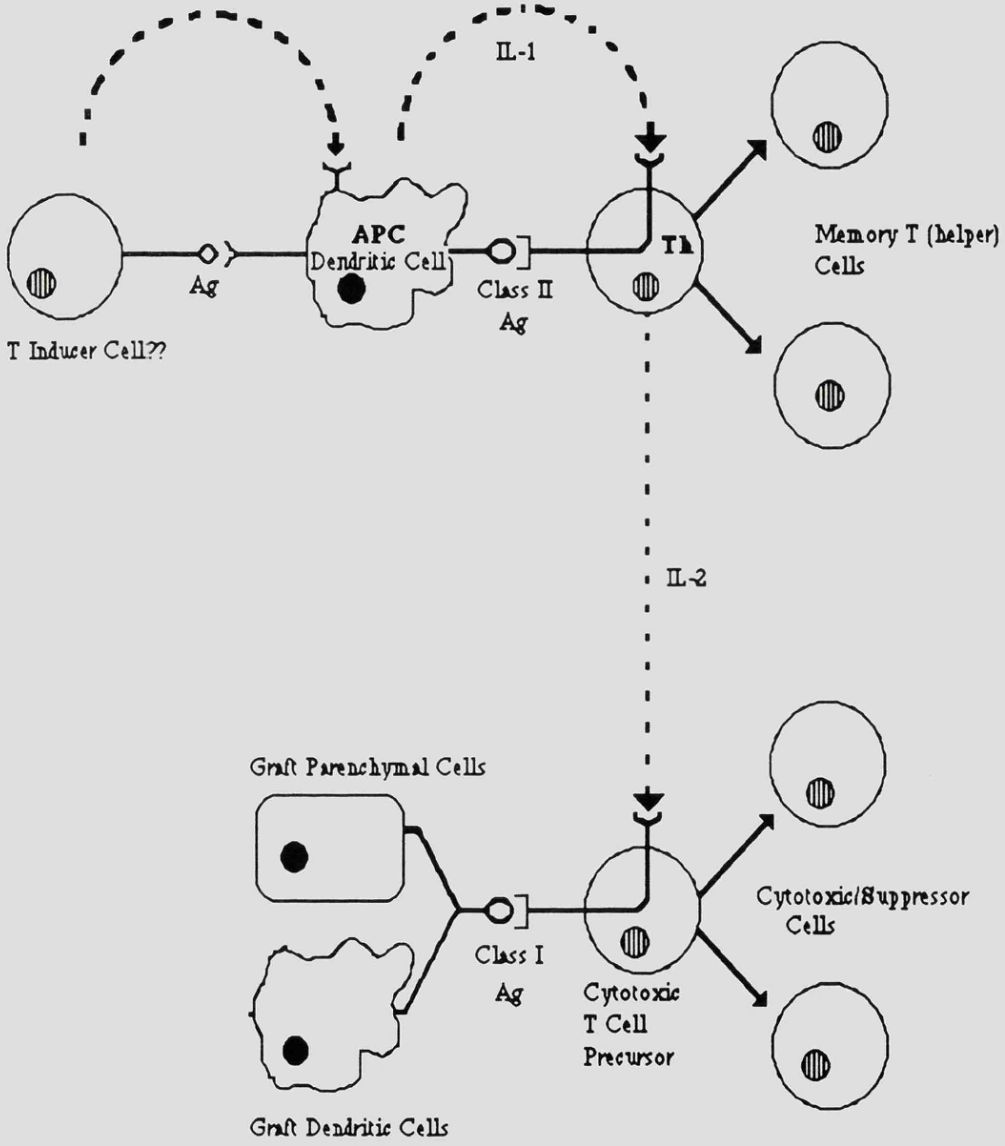

Fig. 11.1 Suggested sequence of events in the activation of T-cells in the transplantation response. Class II MHC antigen is presented to T helper cells (Th). This presentation may be enhanced by the presence of T inducer cells. Macrophages and dendritic cells release interleukin 1 (IL-1) and cause proliferation of the Th cells, and aid differentiation into memory cells. The activated Th cells secrete interleukin 2 (IL-2) which stimulates the T cytotoxic cell precursors (Tc), when bound to Class I alloantigen, to proliferate. *(From Hayry et al, Immunol. Rev., No. 77, 1984.*

donor pretreatment with cyclophosphamide and irradiation support the contention that these DC's were totally inactivated. There was no prolongation of allograft survival when pancreases were transplanted from such donors. The immunohistochemical analysis demonstrated that 12-15% of the Class II positive cells remained in the pancreas at the time of transplantation. The resulting allograft improvement following perfusion must therefore reduce the numbers of these cells even further.

11.3 GRAFT REJECTION AND IMMUNOALTERATION

Allograft rejection has been considered to be one of the immutable consequences of transplanting foreign tissue into non-immunosuppressed recipients. This assumption was based on Medawar's theory that transplantation antigens present on all cells of the graft caused the host to reject the foreign tissue (Gibson and Medawar, 1943). This premise has formed the basis of the two goals of most clinical transplant centres: the development of better methods of matching histocompatability antigens of donors to recipients and the development of improved immunosuppressive drugs to minimize the recipient response to the transplant.

Immunogenicity implies the transformation, generally described as processing, of water soluble native antigen into a membrane-associated protein, or denatured protein fragment, capable of specific interaction with Class II MHC molecules on the surface membrane of antigen presenting cells (APC). Immunoalteration would attempt to inhibit these processes. The fact that the immunoalteration of transplantable tissues is dependent on the 'inactivation' or removal of the Class II positive leucocytes, including APC, from within the allograft suggests that the recognition and subsequent rejection of these pretreated allografts was suboptimal. The effector limb of the rejection process is dependent on the cooperation of different T cell subsets, particularly of the T helper cell being stimulated by appropriate presentation of foreign Class II MHC antigen.

The actual chronological order for the interactions of the cells involved in the immune response has been difficult to ascertain, but several reports have helped clarify some of the basic issues.

that these DC's were totally inactivated. There was no prolongation of allograft survival when pancreases were transplanted from such donors. The immunohistochemical analysis demonstrated that 12-15% of the Class II positive cells remained in the pancreas at the time of transplantation. The resulting allograft improvement following perfusion must therefore reduce the numbers of these cells even further.

11.3 GRAFT REJECTION AND IMMUNOALTERATION

Allograft rejection has been considered to be one of the immutable consequences of transplanting foreign tissue into non-immunosuppressed recipients. This assumption was based on Medawar's theory that transplantation antigens present on all cells of the graft caused the host to reject the foreign tissue (Gibson and Medawar, 1943). This premise has formed the basis of the two goals of most clinical transplant centres: the development of better methods of matching histocompatability antigens of donors to recipients and the development of improved immunosuppressive drugs to minimize the recipient response to the transplant.

Immunogenicity implies the transformation, generally described as processing, of water soluble native antigen into a membrane-associated protein, or denatured protein fragment, capable of specific interaction with Class II MHC molecules on the surface membrane of antigen presenting cells (APC). Immunoalteration would attempt to inhibit these processes. The fact that the immunoalteration of transplantable tissues is dependent on the 'inactivation' or removal of the Class II positive leucocytes, including APC, from within the allograft suggests that the recognition and subsequent rejection of these pretreated allografts was suboptimal. The effector limb of the rejection process is dependent on the cooperation of different T cell subsets, particularly of the T helper cell being stimulated by appropriate presentation of foreign Class II MHC antigen.

The actual chronological order for the interactions of the cells involved in the immune response has been difficult to ascertain, but several reports have helped clarify some of the basic issues.

It was McKenzie's group who demonstrated that graft rejection was restored to adult thymectomised, lethally irradiated and bone marrow restored mice by purified Lyt $1^+2^-3^-$ T cells, but not by Lyt $1^-2^+3^+$ T cells (McKenzie *et al,* 1982). The importance of the obligatory role of T helper cells in allograft rejection was being established. Furthermore, Dallman and Mason showed that similarly prepared rats were restored by W3/25$^+$ T cells but not by MRC/OX8$^+$ T cells (Dallman and Mason, 1982a). In these latter experiments, there was no overlapping of the subsets of T cells and it is these findings which have established that the T helper cell is not only required for graft rejection but it is the pivotal cell in the allo-immune response. It is now known that Lyt 1^+ antigen is common to all T cells (Swain and Dutton, 1980), and thus the findings of McKenzie could be criticised by the purist as mutually exclusive T cell subsets were not used. To further clarify the role of the T helper cell, the Oxford group have shown in elegant experiments that graft rejection can occur when T helper cells *alone* are present (Dallman and Mason, 1982b). This does not mean that other cell types i.e. B cells and macrophages, do not play an important role in allograft rejection.

In recent years, the promising experimental approaches in prolonging graft survival were based on the a theory that depletion in the passenger lymphocyte content of a graft would render the organ less immungenic. The relative importance of immunogenetic elements in an organ allograft remains uncertain, but it is certainly widely accepted that passenger leucocytes or dendritic cells play a major role in not only sensitization, but also initiation of the overall allogeneic rejection response. Stuart and colleagues concluded that graft immunogenicity would be reduced by passenger cell elimination (Stuart *et al,* 1978), but also reported that the vascular component significantly contributes to the induction of the immune response; only a minimal effect could be accomplished when these passenger leucocytes were removed.

However, there may be an alternative to explain the reduction in graft immunogenicity by dendritic cell depletion which minimises the immune induction by non-dendritic cells.

Lafferty proposes that there may be two quite distinct forms of allopresentation that are important for consideration of allograft immunogenicity -

FIG. 11.2

SCHEMATIC DIAGRAM OF CLASS I AND CLASS II ANTIGEN PRESENTATION TO SPECIFIC T CELLS

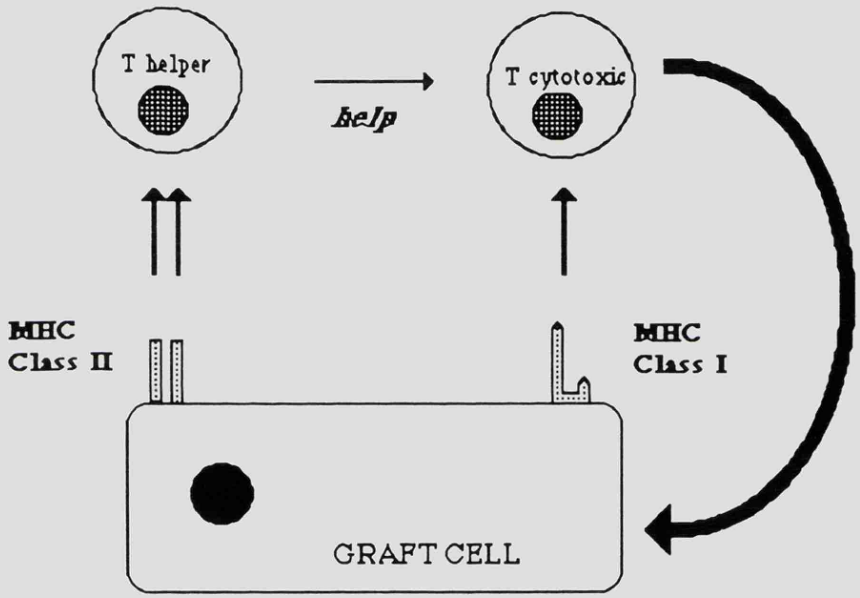

Fig. 11.2 This is a schematic diagram demonstrating the MHC antigen restriction of T helper cells (Th) and T cytotoxic cells (Tc). The presentation of the Class II alloantigen is primarily restricted to the Th cell; the Class I alloantigen is preferentially presented to the Tc cell. Inhibition of the presentation of the Class II antigen produces significantly more inhibition of the alloaggressive response than does the inhibition of Class I presentation.

active and passive antigen presentation (Lafferty *et al,* 1986). Active alloantigen presentation occurs when MHC antigen, on the surface of metabolically (and ? immunologically) active cells is recognised by specific T cells. In this situation, effective activation of alloreactive T cells occurs because these 'active' antigen presenting cells provide both the source of the alloantigen and the inherent stimulatory capacity for immune induction (the so called 'costimulator' (CoS) activity, or signal 2). Lafferty suggests that the antigen presenting cells lacking the stimulator activity can present alloantigen on their cell surface, but cannot evoke T cell responsiveness - passive presentation. In addition to the way in which these antigens are presented, it is the antigens themselves that can be responsible for alloaggresion. Both Ia antigens (Class II MHC antigens) and Class I antigens can elicit an immune response, but it is the expression of the Class II MHC antigens on the surface of the antigen presenting cells, and their subsequent presentation to Class II restricted T helper cells, which is important in evoking an optimal allogeneic response.

The mixed lymphocyte studies performed in the experiments presented in Chapter 7 suggest that host antigen presenting cells can present donor Class II MHC antigen effectively to stimulate the proliferation of T cells. Sherwood *et al* have presented further evidence that this is indeed the case (Sherwood *et al,* 1986). They concluded that alloantigen can be presented by APC's, but interestingly discovered that the effect was only induced by an increase in the numbers of host APC's by a factor of 10 - 100 fold compared to donor APC's. This corroborates the findings in this thesis. 10 - 100 fold of responder-type accessory cells had to be added to the mixed lymphocyte cultures to provoke similar proliferation of T cells compared to stimulator-strain APC's. Perhaps this route of allopresentation *is* less efficient *in vivo* as well, supporting Lechler's and Batchelor's claim that this is indeed the case (Lechler and Batchelor, 1982).

11.4 CONCLUSION

My research demonstrates that rat pancreases can be perfused *ex vivo* for three hours on a normothermic, oxygenated haemoperfusion circuit, and that following perfusion, these organs are viable and can be life sustaining. Isolated pancreases, for example, reversed experimentally induced diabetes in animals

when these pancreases were transplanted following a three hour period of ex vivo perfusion into syngeneic recipients. Similarly, rat kidneys tolerated perfusion well and were able to sustain life function after two and three hours ex vivo perfusion.

The passenger leucocytes, so unique in their capability of inducing an immune response, carry certain transplant antigens (Class II) on their cell surface. The development of monoclonal antibodies, directed against these Class II antigens, has aided the identification of the cells, such that they can be distinguished from other cellular components of the transplantable organs. During *in vitro* studies, these anti-Class II monoclonal antibodies, together with complement, lyse all macrophages and dendritic cells which express Class II antigens on their cell surface. By including these anti-Class II MAb's in the perfusate on the perfusion apparatus, the effects on the survival of these transplant allografts has been studied. It has been clearly demonstrated that pancreatic organ allografts do survive significantly longer following a three hour ex vivo perfusion period, if anti-Class II MAb's are included in the perfusate and the organs are transplanted into non-immunosuppresed recipients. Shorter perfusion periods, of up to one hour, slightly increased the allograft survival time, but simple flushing of the pancreas with the monoclonal antibody had no effect.

Furthermore, the effects of pretreating isolated pancreatic islets on their antigenicity was studied in mixed lymphocyte cultures. This enabled a direct functional assay to be performed on the islets isolated from pancreases perfused for three hours on the ex vivo circuit. It was demonstrated that incubation of islets with anti-Class II MAb and rabbit complement significantly reduced their ability to stimulate proliferation of allogeneic T cells.

Despite the allografts enjoying a prolonged survival following perfusion with anti-Class II MAb, the rejection reaction was not totally abolished in this model and delayed rejection occured, albeit significantly later. In addition, the *in vitro* studies demonstrated that the Class II MHC dendritic cells within a viable organ allograft are non MHC restricted, and that allogeneic dendritic cells can present donor Class II antigen.

I surmise that, even though the immunogenicity of an organ allograft

can be reduced by a pretreatment regimen which removes or inactivates all the MHC antigen positive dendritic cells from within an organ allograft, it is not enough to abrogate allograft rejection altogether. However, the immunogenicity could be reduced sufficiently to allow a reduction in the doses of adjuvant immunosuppressive drugs which have many untoward side effects and are potentially lethal to the transplant recipient.

The recent reports of the success with pancreas transplantation are encouraging. The incidence of allograft rejection episodes, though, remains high and alternative regimens for minimising the drug related morbidity and mortality caused by non-specicfic immunosuppression must be sought. It would be challenging to embark on a clinical study which would assess the efficacy of pretreating transplantable organs with anti-leucocyte common monoclonal antibodies. The results presented by Brewer *et al* are encouraging, since they were able to demonstrate a significant reduction in clinical rejection episodes following pretreatment of human kidney allografts with a cocktail of monoclonal antibodies. A pretreatment regimen utilising ex vivo perfusion of transplantable organs with a cocktail of monoclonal antibodies confers no risk to the transplant recipient and may reduce the level of complications of non-specific immunosuppression and may reduce the severity and frequency of rejection episodes.

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