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UNIVERSITY OF ALBERTA

PREVENTING REJECTION OF HIGHLY-PURIFIED CANINE ISLET ALLOGRAFTS

BY

MARK S. CATTRAL

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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THE UNDERSIGNED CERTIFY THEY HAVE READ, AND RECOMMEND TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH FOR ACCEPTANCE, A THESIS ENTITLED **PREVENTING REJECTION OF HIGHLY-PURIFIED CANINE ISLET ALLOGRAFTS** SUBMITTED BY **MARK S. CATTRAL** IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN EXPERIMENTAL SURGERY.

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Date: October 12, 1990

DEDICATION

This text is dedicated to Erin and our two children Colin and Thomas.

ABSTRACT

Pancreatic islet transplantation is a promising approach for inducing long-term metabolic homeostasis in patients with diabetes mellitus. It is hypothesized that precisely regulated intermediary metabolism provided by an islet graft, will prevent, reverse, or at least stabilize the secondary microvascular complications of diabetes. Currently, clinical trials of islet transplantation are limited by insufficient quantities of islets, and difficulty overcoming immune rejection. Recent advances in the isolation of human and canine islets in our laboratory have allowed us to obtain large numbers of highly purified islets and to identify the critical amount needed to induce normoglycemia. This study focuses on approaches for preventing the rejection of canine islet allografts.

We evaluated the survival of highly purified pancreatic islets transplanted from single canine donors into 31 outbred mongrel dogs immunosuppressed with cyclosporine (CsA) or untreated. Freshly isolated grafts (mean weight ±SE, 0.45±0.04 g containing 119±7x10^3 islets; purity 91% by electron microscopy, n=26) were transplanted into the spleen or renal subcapsular space of four groups of dogs: group 1, autograft-spleen without CsA (5444±688 islets/kg body weight, n=6); group 2, allograft-spleen without CsA (6669±1744, n=4); group 3, allograft-spleen with CsA (8645±1149, n=10); and group 4 allograft-kidney with CsA (10,263±1418, n=6). Islet allografts (10,469±2572, n=5) cultured for 1 week at 24°C were transplanted into the spleen of a final group of dogs treated with CsA, at levels less than those employed in group 3. The CsA was injected intramuscularly daily for 4 days before and 30 days after transplantation. Fasting plasma glucose (PG, mg/dL) and serum CsA trough values (polyclonal RIA) were determined daily. Intravenous glucose tolerance tests (IVGTT) were done before and after transplantation, for calculation of K values (decline in glucose, %/min; preoperatively, mean K=3.9±0.2). Group 1: all 6 dogs were normoglycemic (PG=98±2

and K=1.8±0.2) at 1 month. Group 2: the graft failed in all 4 dogs, at 4±1.2 days. Group 3: all 10 dogs were normoglycemic initially; 4 died (intussusception developed in two and the graft failed at 3 and 9 days in two whose CsA values were <300 μg/L preoperatively), but the other 6 were still normoglycemic when the CsA was stopped at 30 days (mean PG=132±16 and K=0.9±0.2; $p<0.05$ versus group 1). Their CsA values were 708±197 before and 359±41 μg/L during the third week after transplantation; their grafts failed 12.3±3.4 days after the cessation of CsA. Group 4: all 6 dogs were normoglycemic initially but graft failure ensued at 4.3±1.5 days; 3 maintained marginal graft function (PG<225) for 13±2.1 days. At 30 days, 3 of 6 dogs had evidence of graft survival histologically. Groups 5: all 5 dogs were normoglycemic initially; the graft failed at 6, 7 and 11 days in 3, but the remaining 2 were normoglycemic at 30 days (PG=109±9, K=1.4, $p>0.05$ versus group 1). Their mean CsA values before and 30 days after transplantation were 297±57 and 152±15; graft failure occurred at 14 and 23 days after the cessation of CsA.

This data is unique in demonstrating prolonged function of purified allogeneic islets transplanted from individual outbred canine donors. CsA at serum levels >300 μg/L induced prolonged survival of freshly-isolated purified canine islets. Rejection was prompt when CsA was stopped. Glucose tolerance was impaired in islet allograft recipients treated with high doses of CsA. Graft survival is compromised in the renal subcapsular space. Low temperature in vitro culture may facilitate islet allograft survival with reduced CsA immunosuppression.

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ALS	Anti-Lymphocyte Sera
APC	Antigen Presenting Cell
CsA	Cyclosporine A
FCS	Fetal Calf Serum
HBSS	Hank's Balanced Salt Solution
IVGTT	Intravenous Glucose Tolerance Test
IRI	Immunoreactive Insulin
MHC	Major Histocompatibility Complex
PG	Plasma Glucose
PS	Penicillin/Streptomycin
RIA	Radioimmunoassay
TCR	T Cell Receptor
TEM	Transmission Electron Microscopy

CHAPTER 1

INTRODUCTION

Diabetes mellitus, a metabolic disorder caused by an absolute or relative lack of insulin, is manifested principally by hyperglycemia. Exogenous insulin replacement therapy introduced by Banting and Best in 1922, continues to be the mainstay of modern management. Although the life expectancy of diabetics has improved, the development of secondary complications are now responsible for the devastating morbidity and mortality of the disease. Fifty percent of patients with type I diabetes are either blind, in renal failure, or have severe neuropathy by the twentieth year after the onset of the disease (1,2). Up to 40% of new patients admitted to dialysis centers have end-stage renal failure secondary to diabetic nephropathy (3). Diabetes is currently ranked as the fourth leading underlying cause of death in North America.

Mounting evidence supports the hypothesis that the secondary complications of diabetes are caused by imprecise metabolic control (4-7). In 1976, the American Diabetes Association policy statement recommended that physicians strive to "achieve levels of blood glucose as close to those in the nondiabetic state as possible" (7). Many diabetologists believe, however, that this goal is unattainable with current therapeutic modalities (7,8). Moreover, intensifying conventional insulin replacement therapy exposes these patients to the life-threatening risks of hypoglycemia (9). Clearly, new forms of therapy are required.

An appealing therapeutic approach is the replacement of insulin producing tissue by transplantation, to precisely regulate intermediary metabolism. At present two

methods of "total endocrine replacement" (Sutherland DER) are available: 1) whole organ pancreas transplantation; and 2) pancreatic islet transplantation.

PANCREAS TRANSPLANTATION

The concept of pancreas transplantation for treating diabetic patients was first suggested by Minkowski in 1891 (10). During the 1920's Gayet and Guillaumie, and Houssay et al independently demonstrated that the heterotopic pancreas could maintain euglycemia in dogs (11,12). In the late 1950's modern vascular anastomotic suture techniques were applied to experimental pancreas transplantation by Lichtenstein and Barschak, Brooks and Gifford, and Lucus et al (13-15). These and other studies laid the foundation for the initiation of clinical trials at the University of Minnesota in 1966, led by Lillihei et al (16). Although these pioneering studies were dominated by a high incidence of graft loss, patient morbidity, and mortality (17), continuing efforts by several individuals have resulted in remarkable progress in the last two decades (18). In general, advances and developments in pancreas transplantation have been focused on overcoming technical complications related to pancreatic exocrine secretion and vascular thrombosis.

The exocrine component of the transplanted pancreas has been a major source of difficulty since the inception of the procedure. There are two basic approaches for dealing with the exocrine secretions: 1) ductal occlusion; and 2) provision of a conduit for drainage.

Simple ligation of the duct was tried, but soon abandoned, because it commonly precipitated acute pancreatitis, pancreatic abscess and fistula, and was associated with poor endocrine function (17-19). Ductal obliteration by injecting synthetic

plastic polymers into the pancreatic duct has been championed by Dubernard et al (20). While this method eliminates the need for ductal anastomosis, data has emerged suggesting that it may be harmful to long-term endocrine function (21-22). Progressive parenchymal fibrosis is a common histologic feature in these glands, which could be detrimental to islet function and survival (22).

In North America, maintaining ductal integrity has been more popular. In the initial studies by Lillihei et al, the pancreaticoduodenal graft was anastomosed to a defunctioned limb of jejunum (16,17). The duodenal component of the graft seemed to be more susceptible to rejection; anastomotic dehiscence and fistula formation in the duodenum caused much of the morbidity and mortality of these early studies. Indeed, the inability to prevent the rejection of the duodenum was a major factor in the decision by the Minnesota group to transplant segmental pancreatic grafts. Segmental pancreatic grafts also offered the advantages of being technically easier to harvest, and the opportunity to utilize living related donors. However, they are associated with a higher incidence of vascular thrombosis and poorer metabolic control (23).

With the introduction of improved immunosuppressive therapy in the early 1980's, whole organ transplants were reconsidered. It had previously been shown by Aquino et al that the entire pancreas with just a small preampullary button of duodenum could be transplanted successfully (24). Diliz-Perez et al applied this technique in dogs that were immunosuppressed with CsA and demonstrated significantly improved graft survival (25). Starzl et al initiated the clinical revival of whole pancreas transplantation in 1984: a composite graft of duodenum, pancreas, and spleen was transplanted in four patients (23). In the first two patients, approximately 2 feet of jejunum was included

with the graft: it was ultimately resected because extensive mucosal ulceration caused severe protein-losing enteropathy. Improved outcomes occurred in the last two patients when only a small cuff of duodenum was retained.

Until recently the most popular conduit for draining exocrine secretions was a Roux-en-Y limb of jejunum. In the past, free drainage into the peritoneal cavity was also tried; although successful in pigs, dogs and monkeys it frequently caused pancreatic ascites and peritonitis in humans (26-28). The feasibility of using the urinary tract was first shown by Gliedman et al in 1971, when they anastomosed the pancreatic duct to the ureter (29). The disadvantage of this method was the necessity for an ipsilateral nephrectomy at the time of transplantation. In 1984, Sollinger et al introduced the technique of anastomosing the pancreatic duct or duodenum to the urinary bladder (30). This method has been subsequently favored by virtually all major transplant centers in North America and Europe. Its main advantage is the ability to serially monitor graft function noninvasively long-term: graft rejection is frequently heralded by a fall in urinary amylase which allows time to initiate antirejection therapy before the onset of hyperglycemia (31,32).

Vascular thrombosis causes most of the early technical failures in pancreas transplantation. It is often attributed to low blood flow rates in small pancreatic vessels, kinking from a malpositioned graft, or graft pancreatitis. Over the past two decades anticoagulating the recipient, creating arterio-venous fistula between the splenic vein and artery, and transplanting composite grafts of the spleen and pancreas to retain normal vascular hemodynamics have been tried to circumvent vessel thrombosis (23,33). None have eliminated the problem and each carries new risks of morbidity: patients anticoagulated have an increased incidence of postoperative hemorrhage; arterio-venous

fistula predispose to hemorrhagic pancreatitis; and transplantation of the spleen can cause hemolytic anemia, thrombocytopenia, and graft versus host disease (33,34).

When a pancreas transplant has been technically successful it restores normal glucose homeostasis within weeks. Glycosylated hemoglobin soon returns to nondiabetic values and normal glucose tolerance is commonly achieved (35). However, the extent to which pancreas transplantation favorably influences the secondary complications of diabetes is uncertain. Many patients have far advanced lesions, often irreversible at the time of transplantation. Nevertheless, it has been clearly documented that a pancreas transplant will protect a synchronously transplanted kidney from developing diabetic nephropathy (36). It would therefore seem rational to transplant pancreatic allografts in patients who are just beginning to show signs of degenerative complications; but the benefits of this approach would have to be balanced by the risks carried with chronic immunosuppression. Currently, pancreas transplantation is most commonly performed in combination with kidney transplantation in patients who have chronic renal failure secondary to diabetic nephropathy.

As of April 24, 1987, 1157 pancreas transplants had been reported to the International Pancreas Transplant Registry (37). Of these, 892 (76%) were performed after 1982. The overall 1 year graft survival between 1985 and 1987 was 47% and several centers are now reporting 1 year graft survival rates >80% -- a rate that is almost equivalent to cadaveric renal transplants. Despite this remarkable improvement, there is reason to be cautious about its ultimate future application: unlike every other vascularized organ transplant currently performed, the majority of tissue in a pancreas transplant is unnecessary, unwanted, and potentially detrimental.

ISLET TRANSPLANTATION

The disenchantment with pancreas transplantation during the seventies provided the impetus to explore and promote islet transplantation. The feasibility of this approach for treating diabetics was first shown by Ballinger and Lacy in 1972 when they demonstrated improved diabetic control in rats that had received an intraperitoneal islet transplant (38). Since the publication of that landmark study, remarkable progress has occurred, culminating with the recent initiation of clinical trials using highly purified islet preparations (39-41).

Experimental islet transplants can induce long-term normoglycemia and are capable of preventing and reversing the secondary complications of diabetes (42). Compared with pancreas transplantation, islet transplantation offers several advantages which include: 1) technical simplicity of the transplant procedure; 2) available means for long-term graft storage by cryopreservation (43); and 3) the potential of in vitro immunomodulation to promote graft acceptance (44).

Despite ample experimental evidence supporting islet transplantation there has been no long-term clinical success. Wide clinical application of islet transplantation is currently limited by an inability to consistently obtain sufficient quantities of islets to induce long-term normoglycemia, and by the necessity for chronic immunosuppression to prevent graft rejection. Efforts to overcome these two major obstacles will now be reviewed.

TECHNIQUES FOR ISOLATING ISLETS

Early efforts to isolate the islets of Langerhans were primarily motivated by the desire to elucidate their function in vitro. While free-hand microdissection techniques

fulfilled this purpose, the number of islets recovered was limited, and fell short of the quantity required for transplantation (45). The most significant advance in isolation technique occurred with the introduction of collagenase digestion to facilitate the separation of islets from the collagenase framework of the pancreas (46). Subsequently, Lacy et al showed further improvements in islet yield from the rat pancreas by disrupting the parenchyma with the injection of balanced salt solution into the pancreatic duct, before collagenase digestion (47). They also introduced islet purification by density gradient centrifugation with sucrose, which was later supplanted by Ficoll (217). The principles of collagenase digestion and Ficoll density gradient purification continue to be the gold standard for isolating islets from rodent pancreas. Approximately 500 islets can be recovered from a single rat pancreas after purification. The availability of inbred strains of rodents permit the use of multiple donors to acquire sufficient numbers of islets for transplantation.

The islet isolation technique developed in rodents was ineffective when applied to the more compact and dense pancreas of larger mammals (48-50). In 1976, Mirkovitch and Campiche partly solved the problem of low islet yield by eliminating the purification step. These unpurified preparations consisting of 1-2 mm diameter microfragments were shown to reliably reverse diabetes when autotransplanted into the spleen of totally pancreatectomized dogs (51). Subsequent efforts to improve islet yield and engraftment focused on the length of time of collagenase digestion; the type and lot number of collagenase; the technique of mechanical separation; the size of the microfragments; and the optimal site for transplantation (52-59). Others evaluated methods for selectively manipulating the exocrine component with toxic chemicals prior to the isolation procedure to enhance islet yield (60,61). Perhaps the most important refinement in

isolation technique occurred with the introduction of retrograde ductal perfusion with collagenase by Horaguchi and Merrel in 1980 (57). It improved the delivery of collagenase and promoted selective destruction of pancreatic acini with relative sparing of islets. With this method they and others showed substantial improvements in islet yield, engraftment, and function after transplantation (57-59).

Although the microfragment preparation can reverse diabetes reliably when transplanted into the spleen of apancreatic dogs, it has many disadvantages which limit clinical application. First, the large volume of unpurified tissue precludes the use of transplantation sites that have proved effective in rodent models. For example, transplantation of pancreatic microfragments into the liver has resulted in disastrous complications including portal hypertension, disseminated intravascular coagulation, shock and occasionally death (62-64). For dogs, the spleen was found to be the optimal transplantation site because it is large and expansile; the human spleen in contrast would not likely tolerate the assault of unpurified tissue. Moreover, transplanting islets into the human spleen is much more technically demanding because of anatomic constraints not present in the dog. Second, co-transplantation of exocrine tissue may adversely affect engraftment and islet function. This may partly explain the glucose intolerance seen in apancreatic dogs that have received autotransplants of pancreatic microfragments (65-67). Finally, the microfragment preparation is not amenable to pretreatment with immunoaltering techniques designed to facilitate islet allograft acceptance without prolonged recipient immunosuppression (44). Recent studies have also suggested that nonendocrine impurities directly augment graft immunogenicity (68,69).

As the short comings of the pancreatic microfragment preparation were appreciated, the development of an isolation technique that yielded mass quantities of

purified islets became increasingly desirable. To achieve this end, several approaches have been taken to improve the digestion process, methods for separating islets from exocrine tissue, and islet purification.

To optimize collagenase digestion Scharp et al designed a digestion filtration device which permitted complete digestion of the gland and early removal of liberated islets (49). It overcame the problem of relying on subjective assessments to define single end-points of digestion. Several prototypes were subsequently developed for pancreas obtained from a variety of large mammals (70,71). Recently, they have developed an automated method for the human pancreas that allows progressive liberation of islets from the gland (71).

In 1982, Noel et al described a digestion technique that produced sufficient quantities of islets from a single canine pancreas to reverse diabetes in dogs (72). They loaded collagenase into the pancreatic duct and then incubated the gland with collagenase in a shaking water bath at 37°C. At 14 minutes the pancreatic duct was reloaded with more collagenase and the incubation was resumed until general loosening of the tissue was observed. Gentle dissociation of the islets from the exocrine tissue was accomplished by aspiration and injection of the digestate through a graded series of needles, which eliminated the mechanical trauma of mincing, chopping, or grinding that had typified previously used methods. Ficoll density gradient centrifugation was used to purify the islets. Alejandro et al subsequently showed that the injection of the calcium chelating agent EGTA into the pancreatic duct after the first collagenase digestion improved islet yield further (73). With this modification, they were able to reverse diabetes in 17 of 24

dogs with islet autografts implanted into the liver via the portal vein. The purity of the preparation was such that no recipient developed increased portal venous pressure during or after islet implantation.

Noel's technique of distending the pancreatic duct with collagenase has been applied to the human gland as well (39). However, Gray et al found it was essential to increase the calcium concentration in the collagenase solution and to raise its temperature to 39°C (74). After purification they were able to recover 1011 islets per gram of pancreas, with islet purity estimated to be 30%. Scharp et al has also had some success with this method but found it ineffective for pancreas obtained from donors less than 25 years old (75).

Warnock et al combined the principles of collagenase ductal perfusion, gentle manual dissociation, and Ficoll density gradient purification to improve the yield of islets from the canine pancreas (76). This method yielded mass quantities of highly purified islets sufficient to reverse diabetes in totally pancreatectomized dogs when they were autotransplanted into the spleen or liver. Furthermore they defined the critical number of islets necessary to induce prolonged normoglycemia (5000 islets/kg body weight). Later, Rajotte et al and others confirmed the superiority of continuous collagenase perfusion versus collagenase distention of the pancreatic duct for human islet isolation (75,77,78).

Initial efforts to purify islets relied on hand picking (79). While reasonable in rodents, this method is impractical in large animal models and humans where mass quantities of islets are needed. Discontinuous density gradient centrifugation has been the most commonly used technique for purifying islets. Of all the agents evaluated for

this purpose, Ficoll appears to be the most efficient and reliable (50,80,81); although, recent studies by Lake et al suggest that density gradients created with bovine serum albumin may improve islet yield, purity and function (82). The use of density gradient purification is not without problems, however: alteration of the density differences between exocrine and endocrine tissue by organ procurement, storage, and processing can hamper the efficiency of purification.

Other strategies for purification have included fluorescence activated cell sorting, electrophoresis, and magnetic binding of lectins attached to exocrine tissue (50,83). To date none of them have been shown to be any better than density gradient centrifugation.

In the past decade, refinement of isolation techniques have been largely responsible for the remarkable progress that has occurred in islet transplantation in large mammals. Islet isolation from the canine pancreas in particular, has been developed to the point where sufficient quantities of islets necessary to induce prolonged normoglycemia are routinely recovered (76). Nevertheless, further improvements in human islet isolation will be necessary to advance clinical trials. The current shortage of donor organs suggests that all available pancreas including those that may have sustained injury during harvesting and storage must be utilized. Techniques that enable donor organ stratification based on factors that may influence islet recovery must be developed. Furthermore, it will probably be necessary for each laboratory to become familiar with a variety of isolation techniques, such that the optimal technique is selected for a particular type of gland.

ISLET ALLOGRAFT REJECTION

It was originally hoped that free grafts of isolated islets would be weakly immunogenic, as has been demonstrated with other endocrine tissues such as thyroid and parathyroid (84). On the contrary, islets are not only immunogenic but exquisitely sensitive to immune effector mechanisms (85-88), being more quickly rejected than vascularized organ allografts of liver, heart, and kidney, and free grafts of skin, when transplanted across the same histocompatibility barriers (89-92). Although the cause for this finding is not known, several hypotheses as outlined by Perloff et al have been put forth (92): 1) tissue specific islet antigen exists and contributes to augmented immunogenicity; 2) the isolation procedure unmasks islet antigens; 3) the isolation procedure nonspecifically injures islets, rendering them more vulnerable to rejection processes; 4) insufficient islet mass is transplanted such that the loss of only a part of the functioning ß-cell mass results in recipient hyperglycemic and the apparent destruction of the entire allograft; and 5) the method of transplantation of isolated islets as free grafts instead of being immediately vascularized, prejudices their survival.

During the last two decades much attention has been directed towards clarifying and defining the immunologic response to islet allografts. Basic to this process is host recognition of donor antigen, of which the cell surface molecules encoded by the MHC are most important (84). Islet allografts are quickly rejected when transplanted across a MHC barrier, although the exact timing of rejection can be modulated by the reactivity of the donor/recipient strain combination (93,94), the number or mass of islets transplanted (95,96), and the site of islet implantation (97,98). Differences in minor histocompatibility antigens can also contribute to islet allograft rejection, but they are clearly less important than those of the MHC.

MAJOR HISTOCOMPATIBILITY COMPLEX

The MHC, designated H-2 in mice and HLA in humans, is a cluster of genes that occupy 2000-4000 kilo base pairs of DNA on chromosome-17 in mice and chromosome-6 in humans. Four classes of genes map to the complex: class I genes encode for the products H-2-K, -D and -L in mice and HLA-A, -B and -C in humans; class II genes encode for the products H-2, -Ia and -Ie in mice, and HLA-DR, -DQ and -DP in humans; the class III genes encode for products of the complement system; and class IV genes named Qa and T1a in the mouse, encode for proteins that have poorly defined function. The following discussion will be limited to the first two classes of genes.

MHC class I molecules are highly polymorphic glycoproteins consisting of a transmembrane α-polypeptide chain of 338-341 amino acids, that is noncovalently linked to ß2-microglobulin, an invariant polypeptide of 99 amino acids. It is constitutively expressed on all nucleated cells, although the absolute concentration of these antigens show considerable tissue variability. Class II molecules are polymorphic transmembrane heterodimers consisting of an alpha and beta chain each composed of approximately 230 amino acids. The distribution of class II molecules is more limited, being constitutively expressed on cells of the monocyte-phagocyte lineage, dendritic cells, B lymphocytes, and T lymphocytes. Class II antigen expression, however, can be induced in virtually all cells after exposure to interferons (99).

MHC molecules play a dominant role in immunologic processes by determining the recognition of antigen by T lymphocytes. Crystallographic studies of the HLA-A2 molecule by Bjorkman et al revealed that the external domains (α1 and α2) of this molecule fold together to create a single functional domain above which a groove 25Å

long and 10Å wide is formed by two parallel α helices (100). The groove is believed to represent the binding site for processed foreign antigen-peptides; the MHC-peptide complex is then recognized by the TCR as modified self which triggers T-cell activation. Presumably allogeneic MHC molecules structurally resemble self-MHC-foreign peptide complexes.

The binding of MHC molecules with the TCR is determined by the accessory cell surface molecules CD4 and CD8, which are expressed on mutually exclusive populations of peripheral T cells. T cells expressing CD4 recognize foreign antigen in association with MHC class II molecules, whereas T cells expressing CD8 recognize foreign antigen associated with MHC class I molecules. While the precise mechanism by which these molecules influence MHC-Ag-TCR interaction is unknown, it could occur by improving adhesion between APC and T cells, or by facilitating signal transduction (101).

The expression of MHC molecules in nonlymphoid tissue is dynamically regulated and can be influenced by underlying disease states, drugs, infection and circulating immune mediators (99). Probably, the most important MHC regulatory cytokine is interferon-γ which is released by activated T and possibly activated NK cells (102-104). It can up-regulate constitutive expression, and induce aberrant expression of class I and II molecules in a variety of cell types. In rejecting allografts, up-regulation of MHC antigen expression by the local release of cytokines is an integral component of (105-108), but not necessarily synonymous with rejection (109). While the significance of MHC hyperexpression is not fully understood, in vitro data supports the notion that it enhances antigen recognition by alloreactive immunoregulatory and cytotoxic T cells (110,111).

ISLET MHC ANTIGENS

The expression of MHC molecules in islets obtained from a variety of mammalian species has been detailed in several studies (112-116). Islet endocrine cells in the mouse, rat, dog, pig and human express class I antigen but not class II antigens. However, aberrant class II antigen expression may occur: ß cells from patients with juvenile onset diabetes often express class II antigens (117); and rodent and human ß cells express class II antigens after in vitro incubation with interferon-γ (118-121). A small proportion of cells (probably <1%) within islets from mice, rats, and dogs normally express both class I and II antigens. Precise identification of this population of cells has been difficult, but light and electron microscopic morphometry reveal features compatible with macrophages or dendritic cells (114,115). Class II antigen is also expressed on the endothelium of human and porcine islets, the significance of which is unknown.

ISLET IMMUNOGENICITY AND REJECTION

Much of the immunogenicity of islet allografts has been attributed to the sub-population of class II+ cells. According to Lafferty et al, they represent marrow-derived APC which supply the two key signals necessary for activating-immunoregulatory CD4-T cells: signal 1 is allogeneic MHC class II antigen; and signal 2 is an inductive molecule (presumably 1L-1) (122). Support for this passenger-leukocyte concept of alloreactivity comes from a series of studies that have demonstrated prolonged survival of transplanted islet allografts depleted of APC's (44). The variety of immunoaltering treatment protocols that have been used in this regard will be discussed in more detail below. While considerable experimental evidence exists to support this hypothesis, recent studies have indicated that other factors may also contribute to

islet immunogenicity. Markmann et al showed that the fate of transplanted rodent islet allografts depleted of APC's can be adversely influenced by (121) interferon-γ induced hyperexpression of MHC class I molecules. Furthermore, in a subsequent series of experiments they demonstrated a correlation between extended survival of cultured islet allografts and down-regulation of class I expression (123). Stock et al have also demonstrated that pure ß cells can stimulate a cytotoxic T lymphocyte response in vitro, which suggests that class II + cells present in the islets are not absolutely necessary for inducing an immune response in vitro (124).

The effector arm of the immune response to islet alloantigens is mediated by both cellular and humoral processes (125-128). Histologic examination of islet allografts transplanted into the liver demonstrate progressive graft infiltration by mononuclear cells as early as 48 hours after implantation, and by the fourth post-transplant day heavy lymphocytic infiltration is accompanied by recurrent hyperglycemia (125-126). Recent studies by Sutton et al have shown that the effector response is mediated by highly specific cytotoxic T cells (129), a mechanism consistent with rejection processes described for vascularized organ allografts. Humoral antibodies against islet donor antigens can be detected within a week after transplantation. Although they are probably less important than cellular immune processes, they certainly can trigger graft rejection: Naji et al induced islet allograft rejection in 'tolerant' hosts by injecting anti-serum obtained from animals sensitized to donor antigen (127).

OVERCOMING ISLET ALLOGRAFT REJECTION

Approaches available for overcoming rejection of islet allografts can be categorized as follows: 1) minimizing histoincompatibility; 2) reducing graft

immunogenicity; 3) transplanting islets into an immunoprivileged site; 4) recipient immunosuppression; and 5) induction of immune unresponsiveness to donor allo-antigens. This review will specifically address the first four approaches.

TISSUE MATCHING

Successful transplantation of kidneys between identical twins gave clear evidence that matching donor-recipient histocompatibility antigens could profoundly influence the survival of human organ allografts. The discovery and characterization of HLA class I and later HLA class II molecules provided the tools required to match tissues. The vast majority of tissue typing in the past and at present is focused on renal transplantation. In the past, the benefit of minimizing donor-recipient HLA phenotype mismatches has been unclear, particularly as better immunosuppressive agents were introduced and overall graft survival improved. Nevertheless, recent analysis of large multicenter renal transplant registries have shown better long-term organ survival when the number of mismatches were reduced; recipient-donor identity at the HLA-B and -DR loci were shown to be especially important (130).

In experimental islet transplantation minimizing MHC mismatches has also been shown to extend islet allograft survival (84). However, even complete matching of the MHC does not insure indefinite survival: murine islets that are incompatible with the recipient only at the H-Y locus are rapidly rejected. A further concern for islet grafts is the potential for autoimmune destruction following transplantation into type I diabetics. Experimental models of autoimmune diabetes have indicated that effector T cells mediating β cell destruction are MHC-restricted (216). Deliberate mismatching of donor/recipient MHC-antigens would appear to be a logical method for avoiding this problem. While the theoretical importance of matching versus mismatching remains to be

clarified, the short supply of donor organs, in combination with the short preservation time of the pancreas, limits its present application. However, with the advent of islet banking by cryopreservation, it may be possible eventually.

REDUCING GRAFT IMMUNOGENICITY

The notion that in vitro culture could facilitate the acceptance of tissue allografts was first convincingly demonstrated by Lafferty et al when they reported prolonged survival of murine thyroid allografts that had been cultured for 12 days in an atmosphere of 95% oxygen, before transplantation (131). The fact that rejection of these allografts could be initiated by injecting the recipients with peritoneal exudate cells (a source of class II+ APC's) obtained from mice syngeneic to the graft, suggested that immunogenic cells were depleted during culture, but the graft still provided target antigen (132). These and subsequent experiments provided the foundation for Lafferty's two-signal model of alloreactivity that was referred to earlier.

Initial application of this approach to isolated islets failed because islets quickly disintegrate when exposed to 95% 0_2 (133). To circumvent this problem Bowen et al aggregated islets together to create mega-islets, which remain viable even after prolonged exposure to high oxygen concentrations (134). After 7 days of culture in a 95% 0_2 atmosphere, mega-islet allografts have indefinite survival and function in nonimmunosuppressed mice and rats (135,136).

In 1979, Lacy et al reported that in vitro culture of rat islets for 7 days at 24°C in combination with a single injection of antilymphocyte serum resulted in islet survival greater than 100 days (137). This culture approach was based on studies by Opelz and Terasaki which have shown that human lymphocytes cultured for 4 days at 22°C lose

their ability to stimulate mixed lymphocyte reactions (138). While most studies have shown superior reduction of islet graft immunogenicity with 24°C compared with 37°C culture temperatures (123,139,140), others have not (142).

Depletion or inactivation of APCs is believed to be an underlying mechanism by which tissue culture promotes islet allograft survival: injecting recipients of cultured islet allografts with peritoneal exudate cells syngeneic with the graft, induces prompt graft rejection (141). Moreover, MHC class II antigen expression which is thought to be specifically expressed by islet 'dendritic' cells is reduced or eliminated during the culture period (114,142,143). Others have also shown that capillary endothelium, which may have an antigen-presenting capability (144,145), degenerates during culture (146,147). An alternative explanation for the beneficial effect of tissue culture is suggested by Markmann et al's recent demonstration that down-regulation of MHC class I antigen expression is a critical component of culture-induced immunomodulation (123). While the influence of MHC expression on immune processes in vivo is unknown, the reduction of MHC expression may allow engrafted islets to evade immune recognition.

The passenger-leukocyte concept of alloreactivity has had a pervasive influence on islet transplantation. In addition to tissue culture, other in vitro techniques designed to destroy immunogenic cells have been developed and employed with variable success. They include incubation with anti-Ia sera, anti-Ia monoclonal antibody, anti-dendritic cell monoclonal antibody (148-151); irradiation with ultraviolet light (152,153); and cryopreservation (154). Regardless of the specific technique used, one hopes to maximize killing of immunogenic cells, yet retain endocrine cell viability. Unfortunately, in vitro manipulation of islet tissue does entail loss of islet numbers and/or functional integrity, which may be particularly important if the original quantity of islets is only marginal.

Perhaps one solution is to use several synergistic immunomodulating techniques in combination, to minimize endocrine cell loss during the time required to inactivate immunogenic cells.

Although the potential for immunomodulating islet tissue in vitro is well supported by rodent models, there is no direct proof that it will be a valid approach in large mammals or humans. Nevertheless, the observation of prolonged survival of human islet xenografts that were cultured for 1 week before transplantation into mice, provides indirect support (155). Further, preliminary studies of in vitro immunomodulation of purified canine islet allografts by Alejandro et al have shown promising results (156). Whether immunomodulation will completely obviate the requirement of recipient immunosuppression remains doubtful with currently available immunosuppressive regimens (137,156,157). It seems that as the genetic complexity of the animal species increases, the less effective immunomodulation becomes. For example, in contrast with inbred mice, survival of cultured islet allografts in outbred mice is dependent upon recipient immunosuppression with CsA (158). Similarly, immunosuppression, albeit at lower dosage or shorter duration, is necessary for long term survival of cultured rat islet allografts (156,157). In view of these studies, in vitro immunomodulation will unlikely eliminate the need for immunosuppression in large animals or humans, but may reduce the absolute level of immunosuppression required.

Another approach for reducing the immunogenicity of islet allografts is microencapsulation. The capsular membrane would function to prevent interaction with the host's immune system, yet allow free passage of micronutrients to maintain islet viability. During the past decade the development of microencapsulation with porous poly-l-lysine-alginate has been a significant advance (218). Recently, several studies have

demonstrated that encapsulated rat islets will reverse streptozotocin-induced diabetes in nonimmunosuppressed mice (219). These results are promising, but large animal studies are required to determine whether microencapsulation will have a practical application clinically.

IMMUNOPRIVILEGED TRANSPLANTATION SITES

Selecting a transplantation site for isolated islets involves several considerations. A basic requirement for all free grafts of tissue is immediate access to an excellent blood supply, to facilitate nutrient diffusion before neovascularization occurs. The implant site should be safe and should preferably be accessible to enable easy graft retrieval for evaluation. Finally, concern for metabolic efficiency and immunoprivilege is especially relevant for islet tissue. Over the past two decades several sites have been evaluated, but the optimal site remains to be defined.

Although transplanting islets into an intramuscular site is appealing, the results are poor because engraftment is impaired by the limited vascularity of muscle (159). Ballinger and Lacy were the first to show long-term amelioration of diabetes in rats with intraperitoneal implants of islets (38), but consistent normalization of blood glucose in diabetic rats was not obtained until Kemp embolized islets into the liver via the portal vein (160). The superiority of the liver site was shown by the lower number of islets required to induce normoglycemia and the improved long-term function of these grafts. Morphologic evaluation of islets implanted into the liver revealed that they underwent rapid neovascularization in the terminal portal radicals, and formed junctional complexes with contiguous hepatocytes (161,162). In growth of nerve fibers has also been observed, but their functional significance is unknown (161).

In 1977, Finch et al demonstrated the feasibility of implanting islets into the spleen (86). Although they were unable to detect a difference in the blood glucose in diabetic recipients of islets transplanted into the spleen or liver, Feldman et al showed that the biphasic insulin response during IVGTT is significantly more blunted when islets are transplanted into the spleen than the liver (163). This may be partly explained by the observation that insulin released from islets implanted in the liver can escape hepatic clearance (161).

In dogs, the spleen became the favored site of implantation for pancreatic microfragment preparations because it is large and expansile. With the development of purified canine islet preparations the liver and spleen have been both used successfully (76,164), although the long-term metabolic performance of islet autografts seems to be better in the spleen (76). The advantages of the splenic site include portal drainage, rich vascularity, and expendability if the graft had to be removed.

Teleologically, portal drainage of islet grafts would seem essential for optimizing metabolic efficiency. This premise has been validated by several studies that have shown improved glucose tolerance and biochemical profiles in animals with intraportal versus systemic delivery of insulin (165-168). However, others have shown that with a larger ß cell mass, grafts with systemic drainage can induce equivalent metabolic homeostasis (169-171). In view of the marginal islet yields isolated from human pancreas, choosing a site with portal drainage will likely be beneficial.

A curious aspect of islet transplantation is the profound influence the implantation site has on the timing of allograft rejection. Indeed, indefinite or significantly prolonged survival of islet allografts transplanted into the anterior chamber of the eye, brain, and testicle has been reported (172-174). Presumably these sites provide an immunologically

protected environment, but the exact mechanism is not known. Notwithstanding these observations, the practical value of these sites in large mammals and humans is dubious. Currently, the three most commonly used sites for islet transplantation are the liver, spleen, and kidney. Of these, the renal subcapsular space may be immunologically privileged, although the data is conflicting (175-178). Toledo-Pereyra et al reported that a mechanically prepared microfragment preparation of the canine pancreas survives indefinitely in the renal subcapsular space of nonimmunosuppressed allogeneic hosts (178). Yet, others have been unable to observe function of canine-pancreatic microfragment autografts (179,180). Obviously, the immunologic status of the renal subcapsular space is not clearly defined at this time.

RECIPIENT IMMUNOSUPPRESSION

CONVENTIONAL AGENTS

The traditional approach for preventing the rejection of organ allografts is recipient immunosuppression. Until the introduction of CsA into clinical practice in the early 1980's, azathioprine and prednisone were the mainstays of immunosuppressive therapy. When these conventional chemical immunosuppressants were applied to experimental islet transplantation the results were dismal in both rodent (125,181-183) and large animal models (87,88,184).

Of all the agents evaluated before CsA, ALS in isolation (85,181,185,186), or in combination with graft immunomodulation (127,187) was the most reliable for preventing islet allograft rejection. Unfortunately, most of the data for ALS is obtained from rodent models. In the few studies that used ALS in large animals, the results are inconsistent, or obscured by either short follow-up times or by the use of partially inbred animals

(49,188). Inconsistent results may also be partly explained by the polyclonal nature of the preparation which can cause variation in the immunosuppressive potency of different preparations.

The advent of monoclonal antibody technology has provided unprecedented specificity in immunosuppressive therapy, and overcomes many of the problems associated with polyclonal ALS. The monoclonal antibody OKT3 introduced in 1980, specifically binds to CD3, a cell surface molecule associated with the TCR on mature lymphocytes that participates in triggering T-cell activation (189). In clinical organ transplantation, OKT3 has been found to be particularly effective for reversing acute rejection episodes (190,191), and it is now frequently used prophylactically for preventing rejection (192). Presently, the limited availability of a homologous antibody in animal models prevents evaluation in experimental islet transplantation.

CYCLOSPORINE A

CsA, a metabolic by-product of the fungus Tolypocladium inflatum, is a cyclic peptide composed of eleven hydrophobic amino acids. The early studies of Borel et al established that CsA selectively and reversibly inhibited certain subpopulations of immunocompetent cells (193), which were later shown to be T lymphocytes. CsA is not cytotoxic, nor is it myelosuppressive; these unique features distinguish CsA from previously developed chemical immunosuppressive agents. Following the initiation of clinical trials of CsA by Calne et al (194), CsA emerged as the principal immunosuppressive agent for clinical transplantation.

While the influence of CsA on the immune system is now known to be pleotropic, profound inhibition of immunoregulatory T-helper lymphocytes is the crucial element of CsA immunosuppression (195). CsA inhibits the transcription of m-RNA for several

cytokine genes including INF-γ, IL-2, IL-3, IL-4 and IL-5 (196). IL-2 is an essential inductive molecule required for clonal expansion, proliferation, and differentiation of cytotoxic T lymphocytes. The inhibition of other cytokines collectively limit the recruitment, maturation, and proliferation of other cells of the immune system. At higher concentrations CsA may also directly inhibit cytotoxic T cells by interfering with IL-2 receptor expression (197).

The effect of CsA on other cells of the immune system such as macrophages and B-lymphocytes has been difficult to elucidate because the activities of these cells are closely regulated by interaction and communication with T lymphocytes (198). A unique and still unexplained aspect of CsA immunosuppression is the apparent activation and amplification of suppressor T lymphocytes, which occurs during in vitro experiments. This discriminating effect on T lymphocyte subsets has been implicated for facilitating the emergence of immune unresponsiveness to organ allografts in experimental transplantation (197).

The precise mechanism by which CsA blocks gene transcription is unknown, but data suggests that CsA interferes with intracellular signalling processes triggered by antigenic stimuli (199). Recently, cyclophilin, an ubiquitous 17 KD basic cytosolic protein with high binding affinity for CsA, has been shown to be identical to the catalytic enzyme, peptidyl-prolyl-cis-trans-isomerase, which facilitates protein folding during protein synthesis (200,201). It has been proposed that CsA binding to cyclophilin interferes with the formation of transactivation proteins that regulate cytokine gene transcription during T cell activation.

Although CsA has had a tremendous impact on clinical transplantation, being a major factor for the remarkable advancement of liver and cardiac transplantation in

the last decade, it is not without problems. Nephrotoxicity manifested by delayed graft function (renal allografts), elevated serum creatinine, hypertension, hyperkalemia, and renal tubular acidosis frequently occurs in patients receiving CsA. Elucidating the mechanism of CsA induced nephrotoxicity has been difficult because of the wide spectrum of both functional and morphologic changes observed in those kidneys affected. Several theories have addressed direct tubular toxicity, alterations in renal hemodynamics, and platelet aggregation (202). Fortunately, the incidence and severity of this problem has been decreased by employing reduced CsA dosages in more flexible and innovative immunosuppression protocols. Nevertheless, it continues to be a troublesome aspect of CsA immunotherapy, particularly in renal allograft recipients, where it can be difficult to differentiate CsA toxicity from rejection.

CsA is also mildly hepatotoxic; manifested by hyperbilirubinemia. The incidence is variable, and in general correlates with high serum level of CsA. Other toxic side effects of CsA include transient paresthesia, tremors, seizures, hypertrichosis, and gingival hyperplasia.

CYCLOSPORINE A AND ISLET ALLOGRAFTS

Although earlier studies showed that CsA was ineffective with unpurified islet tissue (203), recent data have indicated that it can prolong the survival of purified rodent (204,205) and canine islets (206) and canine pancreatic microfragments (207). Indeed, continuation of immunologic unresponsiveness to an allograft of purified islets after the withdrawal of CsA has been observed in both rats and outbred Beagles (204,207).

Several factors may be contributing to the recent success witnessed with CsA immunosuppression. First, the quality and quantity of the islets transplanted have both

improved: enriching the purity of the islets by hand-picking has been clearly shown by Terasaka et al to facilitate islet allograft survival (205). Second, recent studies have administered CsA parenterally rather than orally, resulting in high blood levels, which appear to be necessary in the dog to prevent the rejection of allografts of purified and unpurified islets (206,207).

Recently, concern has arisen over the potential adverse effects of CsA on islet transplants. Merrel et al reported that autografts of pancreatic microfragments had a much higher failure rate in dogs treated with CsA, and suggested that CsA impaired islet engraftment (208,220). However, studies by other investigators were unable to corroborate their findings (209,210). CsA may also impair islet function; in vitro, CsA directly inhibits the release of insulin from isolated rodent and human islets (211-214). Studies in dogs have also shown that CsA adversely effects insulin biosynthesis and glucose clearance during IVGTT; an effect which may be only partially reversible (215).

SUMMARY

Despite remarkable advances in islet transplantation during the past 15 years, immune rejection continues to be a challenging problem. The recent advances in the isolation of canine islets in our laboratory have allowed us to obtain large numbers of highly purified islets and to identify the critical amount needed to consistently reverse diabetes mellitus. This provides us with a unique opportunity to evaluate several approaches for preventing the rejection of islet allografts in a clinically relevant model. In the chapters that follow we investigate the survival and function of allografts of these purified islets transplanted from single donors into the spleen or renal subcapsular

space of dogs immunosuppressed with CsA. Lastly, we evaluate the potential of immunomodulation of islet tissue by in vitro culture to reduce recipient immunosuppressive requirements.

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CHAPTER 2

TRANSPLANTATION OF PURIFIED SINGLE-DONOR CANINE ISLET ALLOGRAFTS WITH CYCLOSPORINE[1]

INTRODUCTION

Overcoming rejection of islet allografts has been difficult. In rodents this problem has been partly resolved through immunomodulating the graft in vitro: tissue culture (1,2), ultraviolet light (3) and anti-Ia sera (4,5) all facilitate acceptance of islet allografts. Until these techniques have been thoroughly evaluated for applicability in large animals, however, effective immunosuppression will be necessary to promote clinical islet-cell transplantation.

Conventional chemical immunosuppressive agents have been shown repeatedly to be ineffective (6-8). Although earlier studies showed that CsA was ineffective with unpurified islet tissue (9), recent data have indicated that it can prolong the survival of purified rodent (10,11) and canine islets (12) and canine pancreatic microfragments (13). Indeed, continuation of immunologic unresponsiveness to an allograft of purified islets after the withdrawal of CsA has been observed in both rats (10) and dogs (12).

Assessment of the effect of CsA on islet allografts in an outbred large mammal treated with purified single-donor islets has not been reported. Advances in the isolation of canine islets in our laboratory have allowed us to obtain large numbers of highly purified islets and to identify the critical amount needed to consistently reverse diabetes mellitus (14). Now, we have investigated the survival and function of allografts of these

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purified islets transplanted from single donors into the spleen of dogs immunosuppressed with CsA, continuing the evaluation after the cessation of CsA.

MATERIALS AND METHODS

ANIMALS

Thirty-two conditioned outbred unrelated mongrel dogs of either sex, weighing 8-25 kg, were used. The dogs were maintained and cared for in accordance with the recommendations of the Canada Council on Animal Care. Operative procedures were performed under general anesthesia with sodium pentobarbital (30 mg/kg). For recipient dogs, postoperative hydration was maintained with Ringer's solution injected subcutaneously. Water was offered on day 2 and food was increased to a standard full diet supplemented with Cotazym (Organon Canada Inc., Westhill, ON) from day 4. Analgesic (Buprenorphine, Schering, Toronto, ON) was given postoperatively as required. Donor dogs for allograft experiments (n=14) were killed with a bolus overdose of sodium pentobarbital while still anesthetized. Dogs that were to receive their autotransplant or an allograft were maintained under general anesthesia while the graft was being processed.

TOTAL PANCREATECTOMY AND PREPARATION OF GRAFT

In all donors and recipients, through an upper midline ventral incision the entire pancreas was mobilized with all major vascular connections maintained. Cannulae (PE90) were inserted into the right and left branches of the main duct and into the left duct via a cutdown approximately 8 cm from the distal end. The blood vessels were clamped and divided, and the pancreas was removed, weighed and placed in chilled HBSS (Gibco, Chagrin Falls, OH). Islets were isolated with a modification of the technique devised by

Warnock et al (14) (Figure 2-1). Chilled HBSS, 50 mL containing collagenase (Sigma type XI, St. Louis, MO) 0.5 mg/mL, was injected slowly through each of the cannula, which were then attached to a recirculating perfusion apparatus and perfused with chilled HBSS containing collagenase (0.5 mg/mL) at a pressure of 300 mg Hg. Starting at 10 minutes, the temperature of the perfusate was increased slowly to 37°C; the perfusion was continued until the gland became soft and mucoid (approximately 10-12 minutes). The digested tissue was transferred to a beaker containing chilled HBSS, dissociated gently with forceps, washed, resuspended in fresh HBSS and mechanically dispersed by aspiration and injection through graded needles. A small aliquot of tissue was examined periodically under a dissecting microscope until this step was judged complete. The tissue was combined, centrifuged and resuspended in 120 mL of chilled culture medium 199 with Hepes 25 mmol/L (Gibco, Chagrin Falls, OH), 10% FCS, vol/vol (Gibco, Grand Island, NY) and penicillin 100 U/L with streptomycin 100 μg/mL (PS).

For purification of the islets, 4-mL aliquots of tissue suspended in culture medium were placed in 50-mL conical tubes; 4.3 mL of 5X medium 199 and 16.7 mL of Ficoll (density 1.125; Sigma, St. Louis, MO) was added to each tube and 3 5-mL aliquots of Ficoll (densities, 1.085, 1.075 and 1.045) were layered successively over the tissue suspensions (15). The tubes were centrifuged at 550 xg for 25 min at 22°C. Tissue removed from the 1.045/1.075 and 1.075/1.085 interfaces was combined, washed and pelleted. The supernatant was removed leaving only the tissue pellet (Plate 2-1). The weight of the pellet was determined by subtracting the weight of the tube from the combined weight of tissue and tube. The pellet was then resuspended to a final volume of 30 mL in medium 199 with 10% FCS and PS.

EVALUATION OF ISLET PREPARATIONS

A 0.5-mL sample of the graft suspension was combined with 1.5 mL of dithizone (diphenylthiocarbazone, Sigma, St. Louis, MO). At 10 min, when the dithizone had stained pancreatic islets pink (16) (Plate 2-2), 10 aliquots containing 20 μL each were placed on slides and examined with a dissecting stereomicroscope at 25X magnification. Islets with diameters >60 μm were counted, and the number of islets per graft was calculated from the mean number of islets per sample. In an additional sample, the mean diameter of islets was determined by measuring the greater and lesser diameters of 10 randomly-chosen islets with an optical graticule. The total islet volume for each graft was calculated from the mean islet radius using the formula given for a sphere.

Islet purity was evaluated morphometrically by TEM of 6 randomly selected islet preparations. An aliquot of the graft was pelleted and overlaid with liquid 2% agar (Gibco, Madison, WI), care being taken not to mix the tissue to minimize layering of endocrine and non-endocrine tissue. Multiple blocks of the solidified tissue-agar mixture from each islet preparation were cut, fixed in 2.5% glutaraldehyde, and post-fixed in 1% 0s04. Three blocks were selected at random and mounted on 300-mesh copper grids, stained with uranyl acetate and lead citrate. Each block was serially sectioned at three levels for examination in a Philips 410 TEM at 80 KV. The number of grid squares occupied by islet and nonislet tissue were compared to estimate graft purity (Plate 2-3).

TRANSPLANTATION

Defined numbers of purified islets from single donors were refluxed into the splenic veins of individual recipients (17). Group 1 (controls): autograft, no CsA (n=6); the graft contained 5444±688 islets/kg body weight. Group 2, allograft without immunosuppression (n=4); 6669±1744 islets/kg. Group 3, allograft with

immunosuppression (n=10); 8645±1149 islets/kg. For immunosuppression, the dogs were given daily intramuscular injections of CsA dissolved in medium-chain triglyceride oil, for 4 days before and 30 days after transplantation. The target CsA levels were >300 μg/L before surgery and the dose was adjusted to reach levels of 200-300 μg/L by day 30.

BLOOD INDICES

Fasting PG concentration was measured daily postoperatively with a Beckman glucose analyzer (Fullerton, CA). The graft was deemed to have failed on the first of 3 consecutive days when PG rose from initial normoglycemia (PG <150 mg/dL) to >225 mg/dL. Serum CsA was measured by polyclonal RIA (Sandimmune-Kit, Sandoz Ltd., Basle, Switzerland) daily for 3 days before and 14 days after transplantation, and then 3 times a week. IVGTT (0.5 g dextrose/kg) was performed pre- and postoperatively, for which the saphenous veins were cannulated and blood samples for glucose and insulin determinations were taken at 0, 1, 5, 10, 15, 30, 60 and 90 min. The rate of glucose disappearance was calculated as K values (18). IRI concentration was determined by double-antibody RIA (19) with Pharmacia kits and human insulin standards (Uppsala, Sweden).

STATISTICAL ANALYSIS

All results were expressed as mean±SE or median values. Differences in PG after 1 month follow-up were analyzed with the Mann-Whitney U test. The IVGTT data before and after implantation was assessed by analysis of variance. Differences were considered significant when $p<0.05$, unless otherwise specified.

RESULTS

GRAFTS

A mean of 122±8x103 islets, with a mean islet diameter of 118±3 μm, was obtained from 20 pancreases. Mean calculated islet volume was 116±16 μL. Final graft weight was 0.5±0.1 g. TEM morphometry revealed a striking enrichment of islets versus exocrine tissue with a mean composition of 91% islets.

GRAFT SURVIVAL AND FUNCTION (TABLE 2-1)

All 6 recipients of autografts were normoglycemic throughout the study period. The 4 dogs receiving an allograft but with no CsA became hyperglycemic at a minimum of 3 days after implantation. All of the group 3 dogs, recipients of an allograft and CsA injections, were normoglycemic initially. Two of these dogs could not be followed long-term because of intussusception that developed perioperatively; despite careful treatment, one became dehydrated resulting in poor engraftment and mild hyperglycemia (PG=150-250 mg/dL), and the other died of an intestinal infarction. Both dogs had pretransplantation CsA values >300 μg/L which rose to values exceeding 2000 soon after the intussusception began. Six of the remaining 8 remained normoglycemic throughout the 30 days postoperatively when CsA was being administered (Fig. 2-2). In the 6 dogs with initial CsA >300 μg/L, median PG at 30 days was 112 mg/dL which was significantly higher than 98 mg/dL observed in the autograft controls. The mean CsA level of these 6 dogs at 30 days was 219±41 μg/L. Their grafts failed soon after the cessation of CsA - at 12.3±3.1 days (range, 3-23 days); mean CsA value on the day of rejection was 59±11 μg/L. Graft failure occurred at 3 and 9 days in 2 dogs whose preoperative CsA trough levels had been <300 μg/L.

GLUCOSE TOLERANCE TESTING AND PLASMA INSULIN RESPONSE (FIG. 2-3)

Compared with preoperative control values, the autograft recipients had similar fasting PG levels, but K values during glucose tolerance tests had declined from 3.9±0.2 to 1.8±0.2 (not significant). In the immunosuppressed allograft recipients PG was higher and K values had declined significantly to 0.9±0.1. The plasma insulin response was significantly blunted and the peak was shifted to the right in both groups of graft recipients: this was most marked in the allografted dogs, but the reduction was not significantly greater then in autografts.

DISCUSSION

This data is unique for a large mammalian species, showing prolonged survival of allografts of purified single-donor islets transplanted into the spleen employing CsA immunosuppression. All of the surviving dogs had a serum trough level >300 μg/L preoperatively; both of those that had a value <300 μg/L rejected their grafts rapidly. These results concur with those reported by Alejandro et al (12), who transplanted purified islets into Beagles from one or more donors. In all of the long-surviving dogs, we maintained serum trough CsA values at a high level initially but were able to taper the dose to give a mean of 219±41 μg/L by 4 weeks postimplantation. This compares favorably with the studies of Alejandro (12), who maintained concentrations at >300 μg/L (by RIA), and studies in dogs by Kneteman et al (13), in which a level of >600 μg/L (by whole-blood HPLC) was required for pancreatic microfragments. The success with low levels of CsA in the present study may be attributed to the high purity of the islet grafts (>90%), which was confirmed by electron microscopic morphometry in 6 cases and in all

cases by dithizone-specific staining of islets. This purity compared favourably with the 60-90% achieved by Alejandro et al (12) or with pancreatic microfragments in Kneteman et al's series (13).

The prompt failure of the islet allografts after we stopped the CsA contrasts sharply with reports of other recent studies, in which CsA appeared to induce immune unresponsiveness to islet alloantigens (10,12). Dibelius et al (10) reported 90-day mean survival of single-donor rat islet allografts after 3 perioperative intramuscular injections of CsA. In that study, however, the serum CsA level at 70 days was still 90 μg/L - higher than the mean value at the time of rejection in our study (59±11 μg/L). Their results may have related, at least partly, to the persistent CsA levels in serum. In the study by Alejandro et al (12), purified canine islet allograft implanted intraportally survived at least 30 days after the cessation of CsA; in fact, several grafts survived for many months. These authors attributed their success to the high serum CsA levels, which were higher than in our study and may partly account for the difference between the two sets of data. Our results suggest that continued CsA treatment which provides adequate serum trough levels is necessary to ensure continued graft survival.

Other factors that may contribute to immune unresponsiveness to islet alloantigens become apparent when one compares methods used in Alejandro et al's studies (11) and ours. First, they embolized the islet allografts into the liver via the portal vein: the liver may be an immunoprivileged site for islet allografts (20) - although minimal differences have been reported in the survival of intrasplenic and intraportal rodent islet allografts (21,22). Second, they often used multiple donors to provide sufficient islet mass, which may have conferred an immunologic advantage: comparison of the survival of single- and multiple-donor islet allografts of equal mass transplanted

beneath the renal capsule of mice (23) showed significantly longer survival of the latter. The mechanism of this phenomenon is unknown but it may reflect fewer antigen-presenting [illegible] each donor type presented to the recipient. Third, they used beagles, which may account for a species-specific tolerance; our use of outbred mongrel dogs maximized incompatibility. Finally, Alejandro et al. injected the allografts into asplenic recipients (personal communication, March 1988): our animals had an intact spleen, which may make a difference.

The amount of islet tissue we implanted was quantified by counts of the islets and assessment of their average size before transplantation. For allografts, this was >5000 islets of mean diameter 118 μm per kg body weight, the critical minimum that consistently induces normoglycemia after autotransplantation (14). This was done to provide an adequate functioning islet mass and thus distinguish between failure due to rejection and too few islets. In the nonimmunosuppressed recipients, rejection was clearly apparent within 3-7 days when an average of 6669 islets/kg was implanted. When CsA was administered, 8645 islets/kg were provided, in anticipation of the reputed adverse effects of CsA on engraftment (24) and the metabolic function of isolated islets (25). Clearly, neither of these factors caused hyperglycemia as near-normal fasting glucose levels returned during the first week and were maintained throughout the period of immunosuppression, provided the CsA dosage was adequate. Our ability to define adequacy of the islet mass allowed interpretation of results in one dog with adequate CsA levels in which the allograft became unstable after intestinal intussusception: impairment of engraftment was likely due to dehydration or toxicity as a consequence of intestinal obstruction.

Review of the metabolic data showed the fasting PG consistently higher in the long-surviving recipients of allografts than of the autografts. Moreover, GTT revealed poorer performance of these allografts and blunting of their plasma insulin response, findings that are particularly striking in view of the greater islet content (≥60% more than in the autografts). Although this functional impairment may be due to chronic rejection causing progressive loss of islet mass, it may also reflect an adverse effect of CsA on islet engraftment (24) or insulin release: at least in vitro, CsA directly inhibits the release of insulin from isolated rodent (25-27) and human islets (28). It is possible that the CsA serum levels we used were deleterious to islet function, a theory whose proof requires CsA treatment for recipients bearing identical quantities of autografted islets. Kneteman et al's earlier studies of autografts with CsA immunosuppression in dogs demonstrated no adverse effects of CsA on pancreatic microfragments, but in that study the quantity of islet tissue implanted was not defined (13).

In summary, we achieved prolonged function of purified single-donor canine islet allografts transplanted into the spleen with CsA. Further, our data indicates the need for continued immunosuppression to prevent rejection. Precise quantification of the islets allowed us to determine causes of allograft failure and suggested that high trough levels of CsA in the serum adversely affected islet function.

TABLE 2-1

SURVIVAL OF CANINE PANCREATIC ISLET GRAFTS

TRANSPLANTED TO THE SPLEEN

Group	n	Islets transplanted, No./kg body weight (mean±SE)	Graft survival days (mean±SE)
1. Autograft controls; no CsA	6	5444±688	>30x6
2. Allograft; no CsA	4	6669±1744	2,2,6,6 (4±1.2)
3. Allograft; with CsA	10*	8645±1149	2,8 >30x6** (33±6.5)

* Intussusception developed in 2 dogs, causing unstable graft function in one and death of the other.

** The graft failed at 3, 5, 12, 13, 18 and 23 days after cessation of CsA.

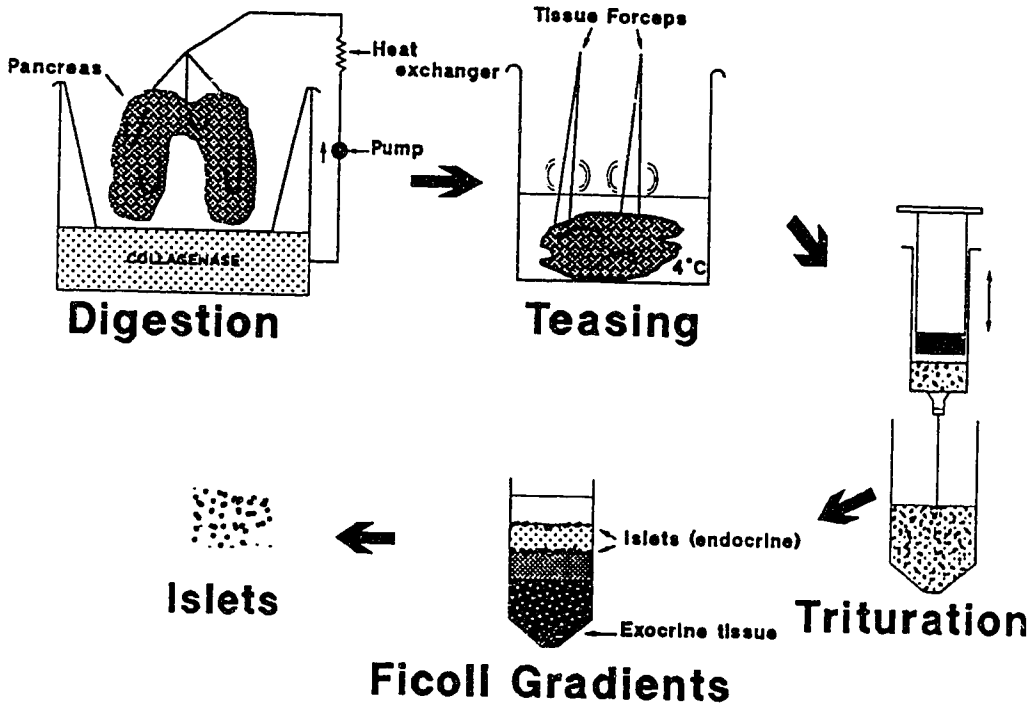

FIGURE 2-1: Schematic of the canine islet isolation procedure.

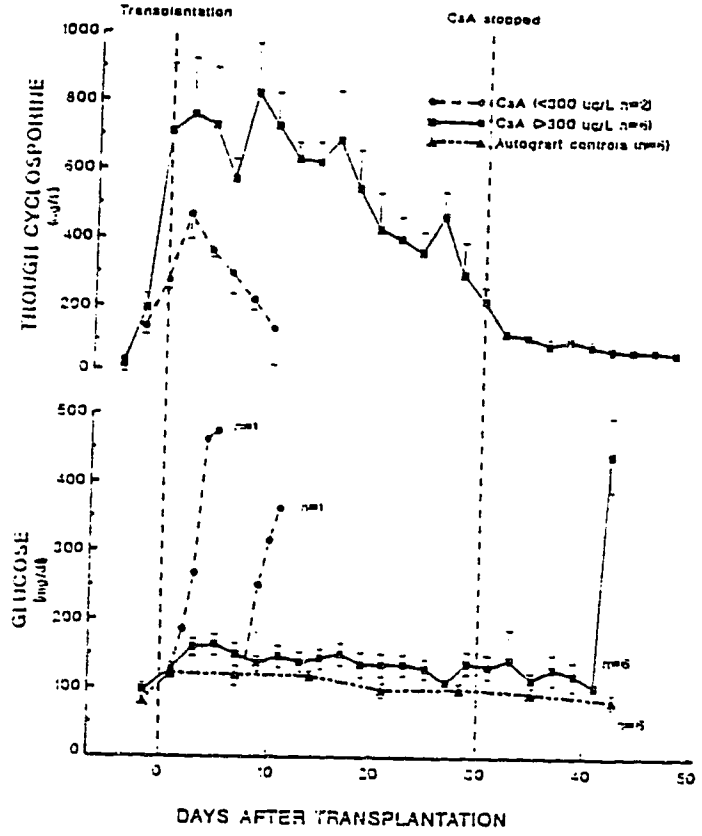

FIGURE 2-2: Mean (±SEM) serum trough CsA levels and fasting plasma glucose concentrations in recipients of islet autografts (group 1) and immunosuppressed recipients of an allograft (group 3, divided according to their CsA level immediately before transplantation - <300 or >300 μg/L).

All 6 autograft recipients were normoglycemic during the study period. The 6 allograft recipients with CsA levels >300 μg before transplantation had prolonged survival but rejected their graft shortly after cessation of CsA. The two allograft recipients with CsA levels <300 μg rejected their grafts at 3 and 9 days.

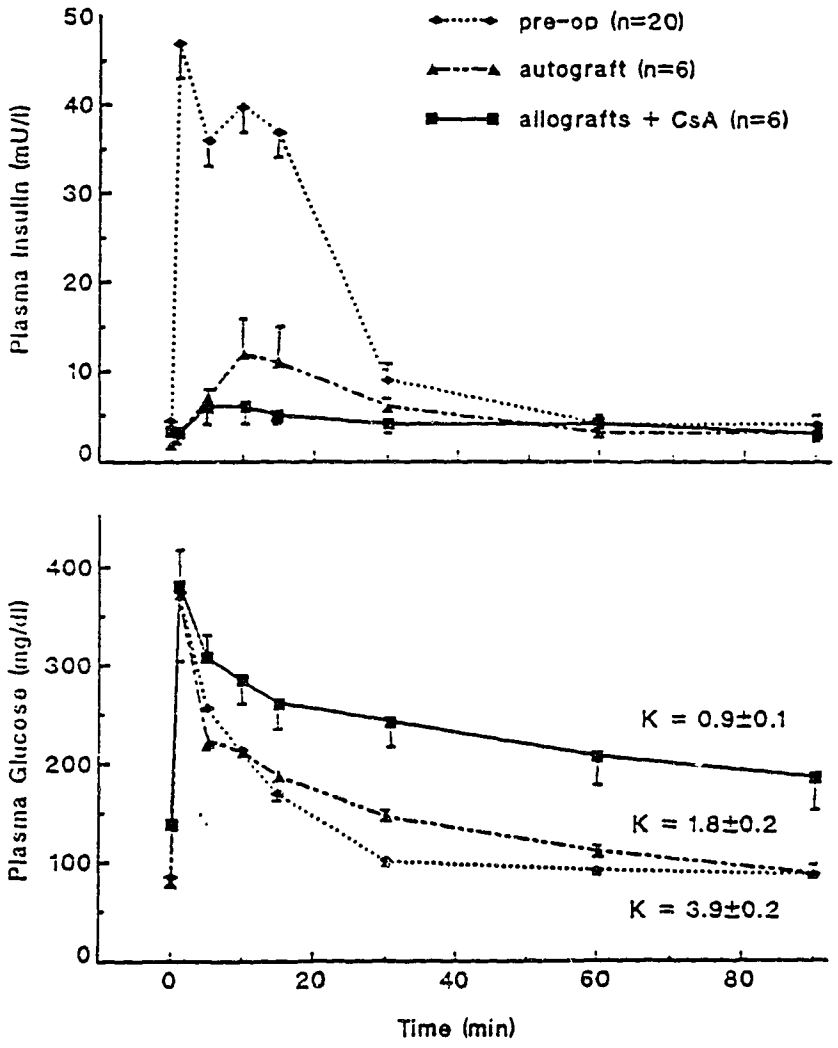

FIGURE 2-3: Plasma concentrations (mean±SEM) of glucose and insulin during IVGTT, pre- and postoperatively, in recipients of islet autografts and long-surviving immunosuppressed allografts.

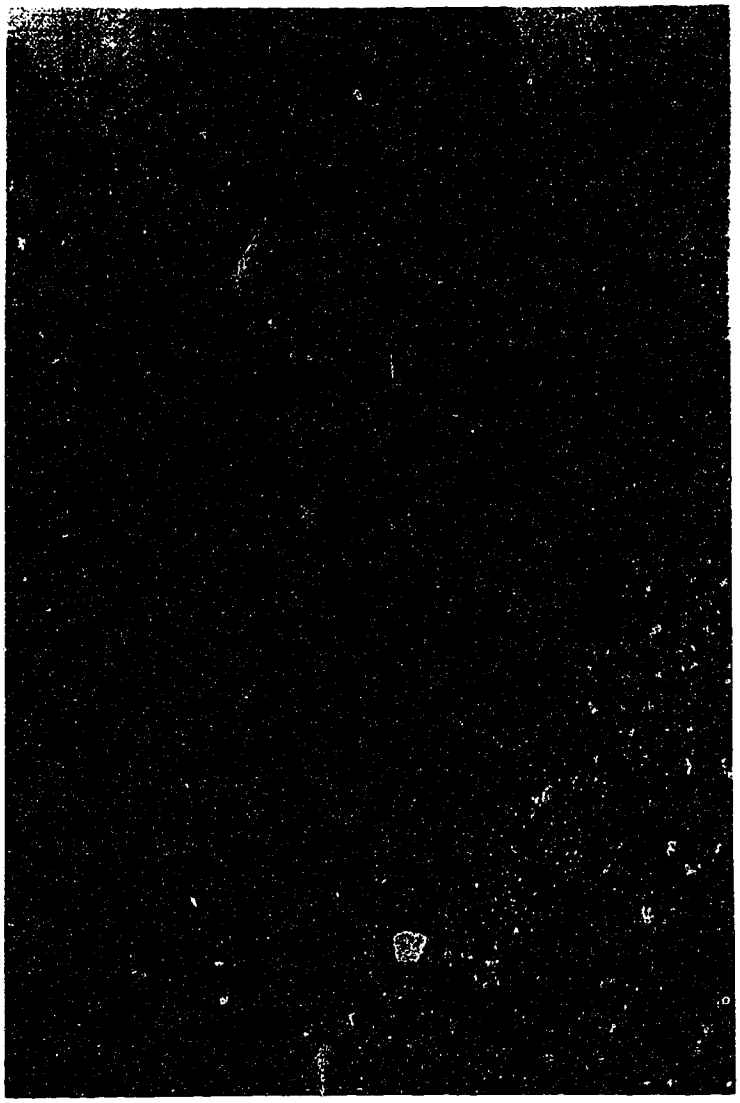

PLATE 2-1: Photograph of an islet graft. The mean final graft weight was 0.5±1 g.

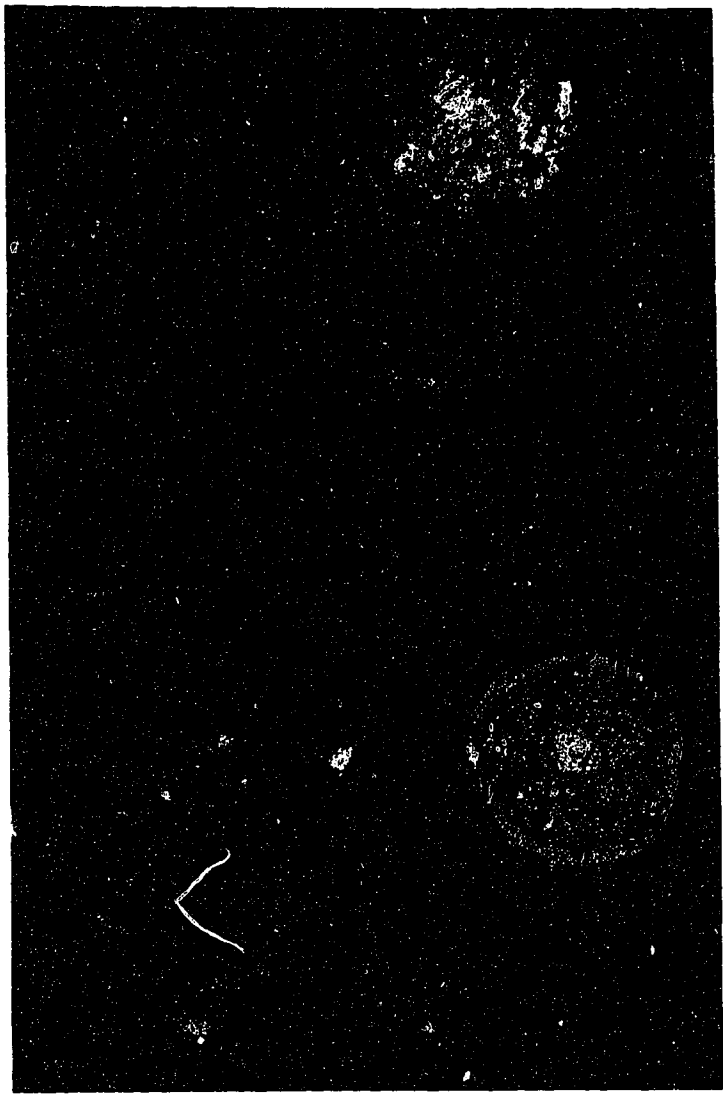

PLATE 2-2: Photomicrograph (incident white-light illumination) of dithizone-stained islets. There is a striking enrichment of islets versus exocrine tissue (original x25).

PLATE 2-3: Transmission-electron micrographs of purified canine islets. A) The number of grid squares occupied by islet and nonislet tissue were compared to estimate graft purity. B) Islets are easily recognized by the characteristic granules in the cytoplasm. (A = original x650; B = original x4700).

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CHAPTER 3

TRANSPLANTATION OF SINGLE DONOR PURIFIED CANINE ISLETS TO THE SPLEEN OR RENAL SUBCAPSULAR SPACE WITH CYCLOSPORINE IMMUNOSUPPRESSION[1]

INTRODUCTION

Islet allograft rejection remains a major obstacle to clinical islet transplantation. Choosing a transplantation site which is immunologically privileged may provide a solution to this problem. Earlier studies in rodent models have shown that the implantation site can profoundly influence the timing of islet allograft rejection. Indeed, indefinite or significantly prolonged survival of islet allografts transplanted into the anterior chamber of the eye, brain, and testicle have been reported (1-3). Presumably these sites provide an immunologically protected environment, but the exact mechanism is not known. Notwithstanding these observations, the practical value of these sites in large mammals and humans is doubtful.

Currently, the three most commonly used sites for islet transplantation are the liver, spleen, and renal subcapsular space. Of these, the renal subcapsular space may be immunologically privileged, but the data is conflicting (4-7). Toledo-Pereyra et al reported that a mechanically-prepared microfragment preparation of the canine pancreas survives indefinitely beneath the renal capsule of nonimmunosuppressed allogeneic hosts (7). Yet, others have been unable to detect function of microfragment autografts implanted into this site. Because islet mass was not quantified in these large animal

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[1]A version of this chapter has been published in Transplant. Proc. 21:2694-2696, 1989.

studies, interpreting the results is difficult. Consequently, the immunologic status of the renal subcapsular space remain undefined in large animals and humans.

With advances in the isolation of canine islets in our laboratory, we can obtain large numbers of highly purified islet and identify the minimum quantity needed to reverse diabetes after transplantation into the splenic site (10). Recent studies have indicated that CsA can prolong islet allograft survival (11,12), and possibly induce a state of unresponsiveness to islet alloantigens (12,13). In the present study we have compared the survival of defined quantities of purified islet allografts transplanted from single donors into the spleen and renal subcapsular space of dogs immunosuppressed with CsA.

MATERIALS AND METHODS

Freshly isolated islet grafts (mean±SE weight = 0.45±0.04 g containing 119±7x10^3 islets, purity 91% by electron microscopy) were prepared as previously described (10). Known quantities of islets were transplanted into 4 groups of totally pancreatectomized dogs: Group 1, autograft-spleen without CsA (5444±688 islets/kg body weight, n=6); Group 2, allograft-spleen without CsA (6669±1744, n=4); Group 3, allograft-spleen with CsA (8645±1149, n=10); and Group 4, allograft-kidney with CsA (10263±1418, n=6). CsA was injected intramuscularly daily for 4 days before and 30 days after transplantation. Fasting PG (mg/dL) and CsA serum trough values by polyclonal RIA were determined daily. The graft was deemed to have failed when PG rose from initial normoglycemia (PG <150) to >225 for 3 consecutive days. IVGTT were done before 30 days and after transplantation, and K values (decline in glucose concentration, %/min) were calculated. For the renal subcapsular grafts, renal vein and arterial plasma insulin concentrations were measured in 5 of 6 dogs during IVGTT; the kidneys were

removed, preserved in 10% formalin, sectioned, and stained with Gomori's aldehyde-fuchsin stain for histologic evaluation.

RESULTS

Table 3-1 summarizes graft survival. All 6 dogs in group 1 (auto-spleen, no CsA) were normoglycemic throughout the study (PG=98±2 at 1 month). In group 2 (allo-spleen, no CsA), hyperglycemia ensued soon after implantation. All dogs in group 3 (allo-spleen with CsA) were normoglycemic initially; 4 died (intussusception developed in 2, and the graft failed at 3 and 9 days in 2 whose CsA values were <300 μg/L preoperatively), but the other 6 were still normoglycemic when CsA was stopped at 30 days (PG=132±16, $p<0.05$ versus group 1). Their CsA values were 708±197 before implantation and 359±41 μg/L during the third week after transplantation; the grafts failed 12.3±3.4 days after stopping CsA. Glucose tolerance in dogs with functioning grafts at 30 days had declined from 3.9±0.2 preoperatively to 1.8±0.2 in autografts and to 0.9±0.2 in allografts. Group 4 recipients (allo-kidney, with CsA), were all normoglycemic initially, but early graft failure ensued (Figure 3-1). The renal capsule was thickened at the site of islet deposition in all recipients. Histologic evidence of graft survival was present in 3 dogs at 4 weeks after implantation: intact islets and dispersed endocrine cells were contained in a loose fibrous stroma associated with minimal lymphocytic infiltration (Plate 3-1). One of these 3 dogs also had an insulin gradient between the renal vein and artery during IVGTT (Figure 3-2). Their mean CsA values before and during the first and second week after transplantation were 623±74, 786±108, and 479±42 μg/L.

DISCUSSION

These data show prolonged function of allogenic purified islets transplanted from single donors into an outbred large animal model. CsA at serum trough values >300 μg/L induces prolonged survival of canine islet allografts, and rejection is prompt when it is stopped. The metabolic performance of long-surviving intrasplenic allografts is impaired during glucose tolerance testing, compared with nonimmunosuppressed autografts.

The duration of function of islet allografts implanted beneath the renal capsule was significantly shorter than for those implanted into the spleen, findings which are striking in view of the greater quantity of islets provided. While functional impairment may be attributed to immune processes causing progressive loss of islet mass, it may also reflect impaired islet engraftment beneath the renal capsule - a theory partly supported by histologic evidence of graft survival in 3 of 6 dogs 30 days after transplantation.

Although the precise cause for poor islet engraftment beneath the canine renal capsule remains to be defined, several factors may be involved. First, the microenvironment of the canine renal subcapsular space may be intrinsically deficient for trophic factors important to islet revascularization. Second, CsA may have contributed to islet loss by reducing or redistributing renal blood flow (14). Third, the diabetic state has been shown to impair blood perfusion of islet grafts (15); we made no attempt to stabilize recipient PG after transplantation with exogenous insulin, which may make a difference. Finally, the unintentional creation of hematoma during graft implantation may have adversely affected engraftment (16).

While sustained normoglycemia did not occur in recipients of renal subcapsular allografts, marginal graft function (PG <225) was evident in 3 of 6 dogs for almost 14

days. This compares favorably with the studies of Hess et al (8) and Evans et al (9) who reported that autografts of canine pancreatic microfragments fail immediately after transplantation beneath the renal capsule. Our improved results may be due to the high purity of our grafts, which minimizes the potential adverse effect of exocrine secretion on islet engraftment. Nevertheless, our results contrast sharply with those reported by Toledo-Pereyra in which mechanically-prepared pancreatic microfragments survived long term, even in nonimmunosuppressed allogeneic recipients (7). However, since no other laboratory has been able to duplicate these results, they should be interpreted with caution.

In summary, these data show prolonged survival of allogeneic purified canine islet allografts with CsA. Further, our data suggests that the function and survival of these grafts is compromised beneath the renal capsule.

TABLE 3-1

SURVIVAL OF CANINE PANCREATIC ISLET GRAFTS TRANSPLANTED TO THE SPLEEN AND RENAL SUBCAPSULAR SPACE

Group	n	Islets transplanted, No./kg body weight days (mean±SEM)	Normoglycemia (mean±SEM)
1. Autograft-spleen; no CsA	6	5444±688	>30x6
2. Allograft-spleen; no CsA	4	669±1744	2,2,6,6 (4±1.2)
3. Allograft-spleen; with CsA	10*	8645±1149	2,8 >30x6**(33±6.5)
4. Allograft-kidney; with CsA	6	10263±1418	1,2,4,10,13,16 (7.7±2.5)

*Intussusception developed in 2 dogs, causing unstable graft function in one and death of the other.

**The graft failed at 3, 5, 12, 13, 18 and 23 days after cessation of CsA.

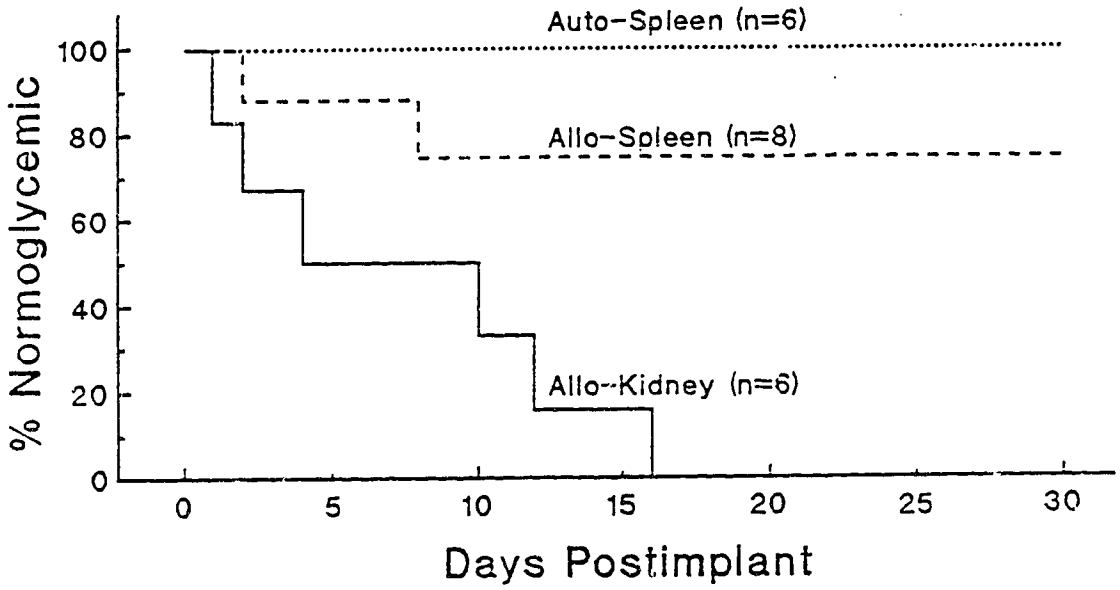

FIGURE 3-1: Percent survival of canine islet autografts, and immunosuppressed allografts transplanted into the spleen or beneath the renal capsule.

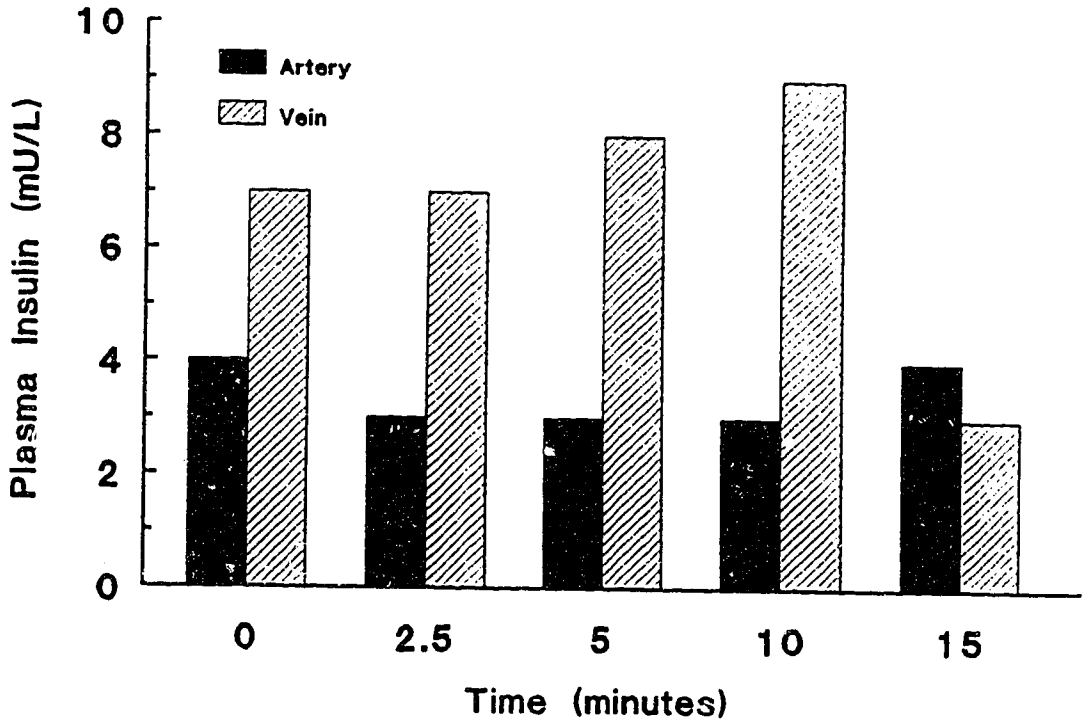

FIGURE 3-2: Renal vein and arterial plasma insulin concentration during IVGTT. One subcapsular islet graft secreted insulin into the renal vein.

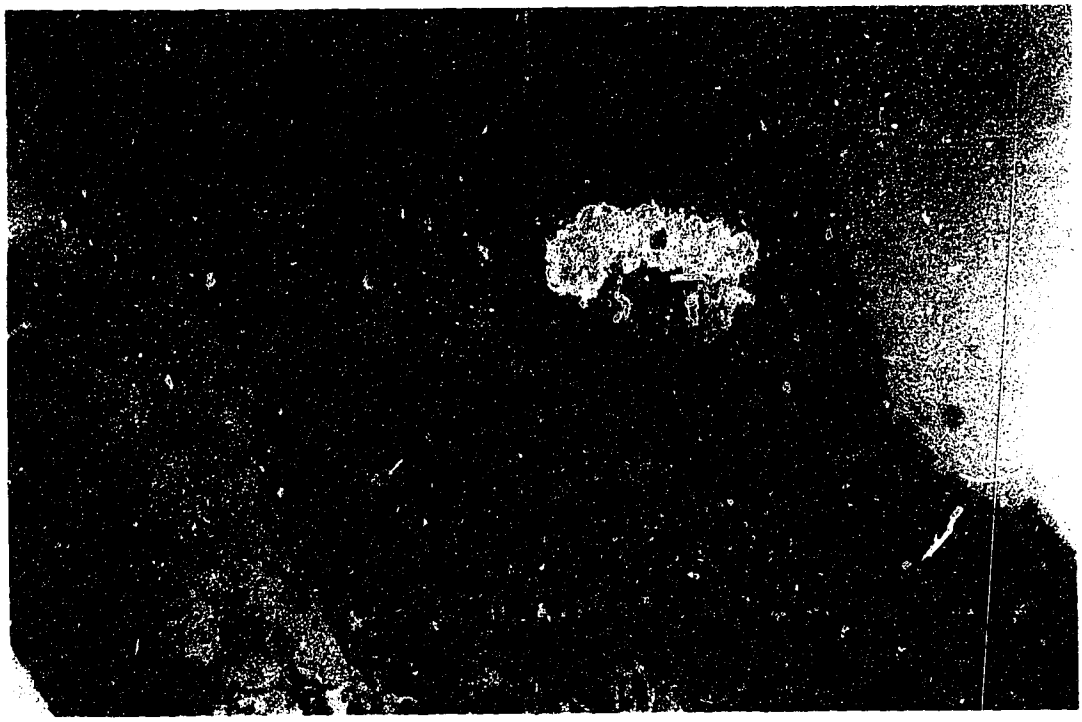

PLATE 3-1: Photomicrograph of islets implanted beneath the renal capsule. Intact islets identified by the purple staining were contained in a loose fibrous stroma (Aldehyde-Fuchsin; original x100).

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CHAPTER 4

TRANSPLANTATION OF PURIFIED CANINE ISLET ALLOGRAFTS AFTER IN VITRO CULTURE (22°C) WITH CYCLOSPORINE

INTRODUCTION

Overcoming immune rejection of islet allografts is necessary to advance clinical trials of islet transplantation. We have shown in an earlier study that CsA can prevent the rejection of highly purified canine islet allografts, but the serum levels required were high (>300 μg/L by polyclonal serum RIA) (1). If these high levels are required clinically, then CsA induced toxicity could potentially be a limiting factor. Therefore, alternative strategies for preventing islet allograft rejection are needed.

In rodents, in vitro culture of islets for 7 days at 24°C facilitates their acceptance in allogeneic recipients (2-4). This approach for immunomodulating islet tissue is presumably mediated by the selective depletion or inactivation of constitutive class II+ APC's (5,6). Previous attempts to validate these results in a large mammalian species have been precluded primarily by the inability to obtain sufficient quantities of purified islets. Recent advances in the isolation of canine islets in our laboratory have allowed us to obtain large quantities of highly purified islets and to identify the critical amount needed to reverse diabetes mellitus (7).

The optimal conditions for maintaining the viability of purified canine islets during in vitro culture have not been reported. The practical application of islet culture in large mammals and humans is contingent upon consistent recovery of the critical islet mass that induces normoglycemia. Accordingly, we performed a comparative evaluation of several

commercially available tissue culture mediums, and two culture temperatures (22°C and 37°C), for their influence on islet recovery and viability. Once the optimal conditions were defined, we evaluated the ability of cultured islets to induce normoglycemia. Finally, we present preliminary evidence that suggests in vitro culture may favorably influence the survival of canine islet allografts.

MATERIALS AND METHODS

ANIMALS

Twenty conditioned outbred unrelated mongrel dogs of either sex, weighing 9-24 kg, were used. The dogs were maintained and cared for in accordance with the recommendations of the Canada Council on Animal Care. Operative procedures were performed under general anesthesia with sodium pentobarbital (30 mg/kg). For recipient dogs, postoperative hydration was maintained with Ringer's solution injected subcutaneously. Water was offered on day 2 and food was increased to a standard full diet supplemented with Cotazym (Organon Canada Inc., Westhill, ON) from day 4. Analgesic (Buprenorphine, Schering, Toronto, ON) was given postoperatively as required. Donor dogs (n=13) were killed with a bolus overdose of sodium pentobarbital while still anesthetized. Dogs that were to receive an allograft were maintained under general anesthesia while the graft was being processed.

TOTAL PANCREATECTOMY AND PREPARATION OF GRAFT

In all donors and recipients, through an upper midline ventral incision, the entire pancreas was mobilized with all major vascular connections maintained. Cannulae (PE90) were inserted into the right and left branches of the main duct and into the left duct via a cutdown approximately 8 cm from the distal end. The blood vessels were clamped and

divided, and the pancreas was removed, weighed and placed in chilled HBSS (Gibco, Chagrin Falls, OH). Highly purified islet grafts were prepared with the isolation technique previously described (1). After Ficoll density gradient purification the islets were washed and pelleted. The supernatant was removed leaving only the tissue pellet. The weight of the pellet was determined by subtracting the weight of the tube from the combined weight of tissue and tube. The pellet was then resuspended to a final volume of 30 mL in CMRL 1066 with 10% FCS vol/vol (Gibco, Grand Island, NY) and penicillin 100 U/mL with streptomycin 100 μg/mL (PS).

IN VITRO STUDIES OF CULTURE ISLETS

CULTURE

Defined quantities of freshly isolated islets were transferred to 100 mm-diameter uncoated plastic petri dishes containing 10 mL of culture medium with FCS and PS (Plate 4-1). The culture media evaluated were: 1) RPMI 1640 without hepes; 2) RPMI 1640 with hepes (25 mmol/L); 3) medium 199; 4) HAM'S F-12; and 5) CMRL 1066. The petri dishes were incubated in a humidified atmosphere of 95% air:5% CO_2 at room temperature (22-24°C) or 37°C for 1 week (Plate 4-2). Culture medium was changed on the third and sixth day. For perifusion studies, islets that had been cultured at room temperature were transferred to the 37°C incubator after the final media change. On the seventh day of culture, the islets were harvested, combined, and resuspended in a known volume of medium with FCS and PS. Samples of the cultured islets were stained with dithizone, and assessed for size and quantity (see below).

PERIFUSION

Islet viability was assessed by insulin release during perifusion. Defined quantities of cultured islets were thoroughly suspended in medium and duplicate samples were transferred to the perifusion chambers. The islets were perifused with Kreb's (Flow; McLean, VA) at 37°C containing glucose as follows: initially 50 mg/dL glucose for 60 minutes, then 500 mg/dL for 60 minutes, and finally 50 mg/dL for 60 minutes. The effluent from the chamber was sampled at ten-minute intervals, with additional samples being taken at 1, 2.5, 5 and 7.5 minutes after the change to high glucose. The samples were collected and insulin concentration was measured by double-antibody radioimmunoassay with Insulin RIA Kits (Pharmacia; Uppsala, Sweden) and human insulin standards (World Health Organization International Lab for Biological Standards).

HISTOLOGY

Aliquots of culture islets were pelleted and overlaid with liquid 2% agar (Gibco; Madison, WI). Multiple blocks of the solidified tissue-agar mixture were cut, fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for histologic evaluation.

ISLET TRANSPLANTATION

CULTURE

Defined quantities of freshly isolated islets were transferred to 100 mm-diameter petri dishes (approximately 5000 islets/dish) containing 10 mL of CMRL 1066 with FCS and PS. They were maintained in a humidified atmosphere of 95% air:5% CO_2 at room temperature for 1 week. The culture medium was changed as described above; HEPES soltuion (25 mmol/L) was added to the medium on the final change. On the seventh day

of culture, the islets were harvested, combined, weighed, and resuspended in 30 mL of CMRL 1066 with FCS, PS, and HEPES.

GRAFT EVALUATION

Before and after tissue culture, a 0.5-mL sample of the graft suspension was combined with 1.5 mL of dithizone (diphenylthiocarbazone, Sigma, St. Louis, MO). At 10 minutes, when the dithizone had stained the islets pink (8), 10 aliquots containing 20 μL each were placed on slides and examined with a dissecting steromicroscope at 25X magnification. Islets with diameters >60 μm were counted, and the number of islets per graft was calculated from the mean number of islets per sample. In an additional sample, the mean diameter of islets was determined by measuring the greater and lesser diameters of 10 randomly-chosen islets with an optical graticule.

TRANSPLANTATION

Defined numbers of cultured islets obtained from single donors were refluxed into the splenic veins of individual recipients (9). The grafts contained 10,469±2572 islets/kg body weight. For immunosuppression, the dogs were given daily intramuscular injections of CsA dissolved in medium-chain triglyceride oil, for 4 days before and 30 days after transplantation. The target serum CsA levels were 150-250 μg/L before and after transplantation.

FOLLOW-UP

Fasting PG concentration was measured daily postoperatively with a Beckman glucose analyzer (Fullerton, CA). The graft was deemed to have failed on the first of 3 consecutive days when PG rose from initial normoglycemia (PG <150 mg/dL) to >225 mg/dL. Serum CsA was measured by monoclonal RIA (Sandimmune-Kit, Sandoz Ltd., Basle, Switzerland) daily for 3 days before and 14 days after transplantation, and then

three times a week. IVGTT (0.5 g dextrose/kg) was performed pre- and postoperatively, for which the saphenous veins were cannulated and blood samples for glucose and insulin determinations were taken at 0, 1, 5, 10, 15, 30, 60 and 90 minutes. The rate of glucose disappearance was calculated as K values (10). IRI concentration was determined by double-antibody RIA (11) with Pharmacia kits and human insulin standards (Uppsala, Sweden).

STATISTICAL ANALYSIS

All results were expressed as mean±SE. Differences in islet quantity before and after culture were analyzed by paired Student T-tests. Differences were considered significant when $p<0.05$.

RESULTS

THE EFFECT OF CULTURE ON ISLET RECOVERY AND FUNCTION

In the initial series of experiments the recovery and insulin-secretory response during perifusion of islets cultured in RPMI, RPMI with hepes, medium 199, and HAM'S F-12, at room temperature or 37°C were compared. As shown in table 4-1, there was a decrease in the quantity of islets during 1-week of culture regardless of the medium or culture temperature used. Nonetheless, there was a trend that favored those cultured at room temperature which was consistent across all types of media. The results of perifusion are shown in figure 4-1. With the exception of islets cultured in HAM'S F-12, islets cultured at 37°C responded promptly with a 4- to 5-fold increase of the insulin secretory rate, constituting a first secretory phase, which was followed by a fainter second phase. In contrast, the insulin secretory response of islets cultured at room temperature was blunted.

Morphometric evaluation showed that islets became more spherical and compact during the culture period, but their size remained constant. However, for islets with larger diameters (>125 μ), central necroses was commonly seen in those cultured at 37°C (Plate 4-3).

Guided by these results two islet grafts were cultured in RPMI with hepes for 1 week at room temperature, but the quantity of islets remaining was insufficient to induce normoglycemia.

I next compared RPMI with hepes and CMRL 1066, the results of which are shown in table 4-2. In both experiments, islet recovery was clearly superior with CMRL. The dramatic loss of islets cultured in RPMI with HEPES is difficult to explain, but it may reflect the poor quality of the islets (in both experiments the islets used were small and fragmented before culture).

The final 7 grafts were cultured in CMRL with FCS and PS at room temperature for 1 week. The number of islets before and after culture, and the percent recovery are shown in table 4-3. Mean islet diameter before and after culture was 125±8 and 117±4 (not significant). The mean graft weight declined significantly during culture from 0.49±0.12 to 0.31±0.07 g. These culture conditions provided consistent recovery of the critical amount needed to induce normoglycemia and enabled the evaluation of transplanted cultured islet allografts.

FUNCTION AND SURVIVAL OF CULTURED CANINE ISLET ALLOGRAFTS (TABLE 4-4)

Of the 7 recipients of cultured islet allografts, 2 dogs could not be followed long-term: technical difficulties during the transplant procedure resulted in graft loss in one; and the other died shortly after surgery because of anesthetic overdosage.

Normoglycemia rapidly ensued after transplantation in the remaining 5 dogs and was maintained until 6, 7, and 11 days in 3 dogs and for the duration of CsA therapy in 2 dogs. In these two latter dogs mean PG at 30 days was 109±9 (Figure 4-2). Their mean serum CsA value on the day of transplantation was 293±57 and by day 30 it had declined to 152±15 μg/L. Their grafts failed at 14 and 38 days after the cessation of CsA. In those dogs with early graft failure, the pretransplant CsA value was 142 μg/L in one, while the two others had CsA values >300 μg/L immediately after transplantation: in order to maintain the CsA values within the target range, CsA injections were withheld for 2 and 4 days respectively during the first week after transplantation.

In an earlier study of fresh islet allografts, 2 dogs with pretransplant CsA values <300 μg/L rejected their grafts at 2 and 9 days (Figure 4-2). Subsequent studies in this laboratory have shown that fresh islet allografts in combination with low levels of CsA results in uniform graft failure by 15 days — mean 9.2±2.6 days (26).

GLUCOSE TOLERANCE TESTING AND PLASMA INSULIN RESPONSE (FIGURE 4-3)

Compared with preoperative control values, the two graft recipients with prolonged survival had similar PG values but K values during glucose tolerance tests had significantly declined from 3.9±0.2 to 1.4±0.1. The plasma insulin response was significantly blunted and the peak was shifted to the right in both recipients.

DISCUSSION

The primary objective of this study was to determine whether 7 days of low-temperature culture of purified canine islet allografts would render them less immunogenic, such that the dosage of CsA necessary to prevent their rejection could be

reduced. Prolonged graft survival was seen in 2 of 5 dogs immunosuppressed with serum CsA levels that were less than the critical minimum required to prevent the rejection of fresh islet allografts (1). These results suggest that tissue culture may reduce graft immunogenicity, but the effect is inconsistent, as shown by early graft failures in the remaining 3 dogs. When the CsA was stopped at 30 days, the two grafts promptly failed. This indicates that 1 week of tissue culture did not eliminate target islet antigens, nor did it result in the development of an immune unresponsive state to islet antigens in combination with low level CsA immunosuppression. These results contrast sharply with those reported by Teresaka et al who showed that low temperature culture in combination with 3 days of CsA therapy induced prolonged survival of rat islet allografts (12).

The inability to consistently prolong survival of cultured canine islet allografts in recipients treated with low level CsA immunosuppression is not readily explained, but several factors may be involved. Inadequate pretransplant serum CsA levels may have contributed to early graft failure in one dog, but the other two actually had serum CsA values that exceeded the target range during the first week after transplantation. Predicting the degree of recipient immunosuppression solely on the basis of CsA serum values may also be misleading: intracellular concentrations of CsA may not reflect serum values; and an individual's response to a given CsA level may vary. Given this difficulty, larger numbers of treated and control animals need to be evaluated to enable statistical evaluation of the impact of culture on islet allograft survival. Another factor may be that islets from large mammals are more resistant to in vitro treatment protocols designed to deplete immunogenic cells - a theory partly supported by studies of Alejandro et al who showed that canine islet allografts incubated with anti-Ia monoclonal antibody plus complement also failed early (13). It may in fact be necessary to combine several in vitro

treatment protocols to reduce islet immunogenicity sufficiently in large mammals (13,14). Another factor that may have contributed to early graft failure is unintentional up-regulation of islet allo-antigen expression secondary to non-specific injury, which could render the grafts more susceptible to immune processes. Finally our use of a large outbred animal model may have influenced our results by maximizing incompatibility. This concurs with reports of other recent studies, in which in vitro immunomodulation became less effective as the reactivity of the donor-recipient combination increased (13,15).

The quantity of cultured islet tissue implanted into each recipient in this study exceeds the critical minimum that consistently reverses diabetes mellitus in apancreatic dogs after autotransplantion of fresh grafts (7), but was comparable to the quantity used in studies of fresh allografts (1). This was done to allow for the possible reduction of functional integrity of cultured islets, and in anticipation of the reported adverse effects of CsA on insulin biosynthesis and islet engraftment (16-19). The rapid induction of normoglycemia in all transplant recipients indicates in vitro culture does not have an adverse effect on immediate islet function and suggests that sufficient islet mass was implanted into each dog. While the three early graft failures could be attributed to a deleterious influence of culture on islet survival, acute rejection was deemed a more likely cause. However, evaluation of the survival of identical quantities of cultured islet autografts would be necessary to prove this theory.

Review of the metabolic data in the 2 dogs with graft function exceeding 1 month, revealed a reduction in the rate of glucose clearance and stimulated insulin release compared with control values. Nevertheless, these results compare favorably with those seen in our previous study of fresh islet allografts treated with higher dosages of

CsA (1) (Figure 2-3). These results suggest that the improved glucose clearance in the present study may be due to the lower levels of CsA employed and are in agreement with the results of studies by Van Schilfgaarde et al who showed that the adverse affect of CsA on in vivo insulin secretion in dogs correlate with the CsA blood level (20).

The results of these studies demonstrate the feasibility of long-term storage of highly purified canine islets by low-temperature tissue culture. After 7 days of culture the number and the size of the islets decreased insignificantly. However, the aggregate graft weight did decrease significantly during culture which suggests that nonendocrine impurities may have been selectively depleted. The ability of in vitro tissue culture to enrich the purity of islet grafts has been previously reported (21). Islet morphology was well preserved during culture, which compares favorably with other studies that have commonly observed central necrosis in islets subjected to 1-week culture (22,24,25). Functional integrity of the cultured islets was also well maintained as shown by the rapid induction of normoglycemia in all graft recipients. These results concur with those of other studies that have shown that low-temperature cultured islets provides sufficient function to reverse hyperglycemia within 24 hours after transplantation (22,24).

The high rate of islet recovery in this study may be partly explained by our use of a low culture temperature. In vitro data suggested that the yield and morphologic appearance of canine islets was better after 1 week of culture at room temperature versus 37°C, which concurs with previous studies of human and rat islets (22,24). The beneficial effect of a lower culture temperature on islet survival is probably mediated by reducing their metabolic activity, and consequently their requirements for substrates, such as oxygen, which may be in limited supply. Islet recovery also appears to be influenced by the composition of the culture medium. Our data demonstrates that CMRL is clearly

superior at maintaining the morphologic and functional integrity of purified canine islets. These results contrast sharply with those of Andersson et al, who reported that RPMI in combination with a culture temperature of 37°C were the most favorable conditions for long-term storage of murine islets (23). In that study, however, only in vitro evaluation of islet function was performed, which may not correlate with islet recovery or function after transplantation (25). The efficiency of our culture conditions provided a unique opportunity to evaluate the survival of cultured islet allografts transplanted from single donor pancreas.

In summary, this preliminary study of the effect of low temperature culture on canine islet allograft survival suggests that it may be beneficial. Further studies are clearly required to confirm these observations and to define alternative culture protocols which can induce superior or consistent reduction of graft immunogenicity.

TABLE 4-1

PERCENT RECOVERY OF PURIFIED CANINE ISLETS CULTURED FOR 1 WEEK IN DIFFERENT MEDIA AT 22 VERSUS 37°C

	Medium:	RPMI		RPMI with HEPES		Medium 199		HAM'S F-12	
Experiment	Temperature:	37°C	22°C	37°C	22°C	37°C	22°C	37°C	22°C
H031		83	94	--	--	76	126	--	--
H322		23	43	25	37	31	48	35	40
H231		73	91	87	151	62	155	58	156

TABLE 4-2

PERCENT RECOVERY OF PURIFIED CANINE ISLETS CULTURED FOR 1 WEEK IN CMRL VERSUS RPMI WITH HEPES AT 22°C

Experiment	CMRL-1066	RPMI with HEPES
H374	36	0
H388	59	0

TABLE 4-3

NUMBER OF ISLETS BEFORE AND AFTER 1 WEEK OF CULTURE

IN CMRL 1066 AT 22°C

Experiment	Before Culture	After Culture	Percent Recovery
H476	180,000	116,000	64
H518	108,000	109,000	101
H512	102,000	98,000	96
H547	137,000	131,000	96
H546	93,000	123,000	132
H594	231,000	216,000	94
I007	104,000	85,000	82
mean±SE	136,613±19,383	125,340±16,194	95±7

TABLE 4-4

SURVIVAL OF CULTURE CANINE PANCREATIC ISLET GRAFTS

Group	n	Islets transplanted no./kg body weight mean±SEM	Graft survival days mean±SEM
Cultured-allograft	7*	10,469±2572	6,7,11,44,68
Fresh-allograft (control)	2	8176±2062	2,8

* Technical complications caused the death of 2 dogs early after transplantation.

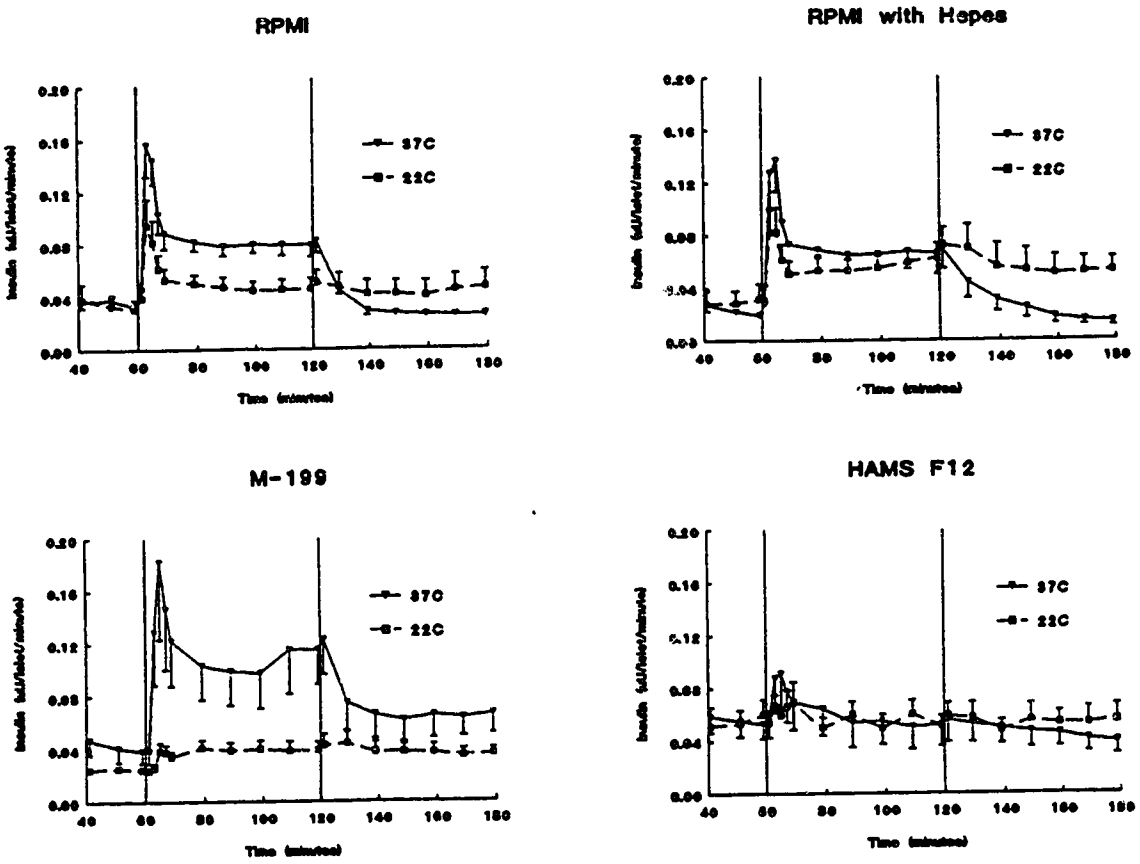

FIGURE 4-1: Functional activity during perifusion of purified canine islets cultured for 1-week in different media at 22 or 37°C. The glucose concentration in the perifusion media during the three 60-minute time intervals was 50, 500 and 50 mg/dL respectively.

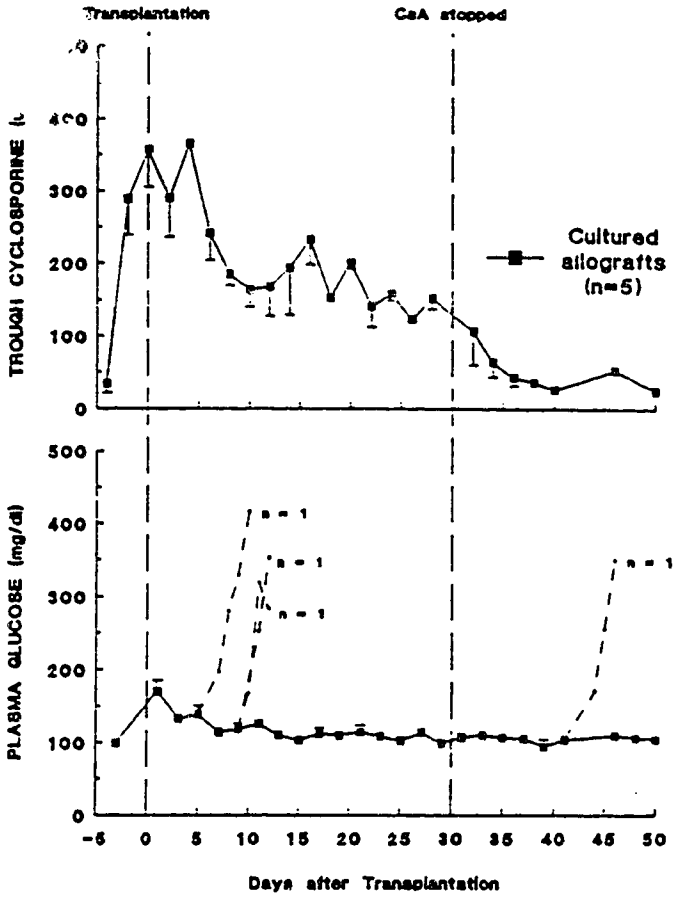

FIGURE 4-2: Mean (±SEM) serum trough CsA levels and fasting plasma glucose concentrations in recipients of cultured islet allografts immunosuppressed with low levels of CsA (<300 µg/L immediately before transplantation). Two recipients had prolonged survival but rejected their grafts shortly after the cessation of CsA.

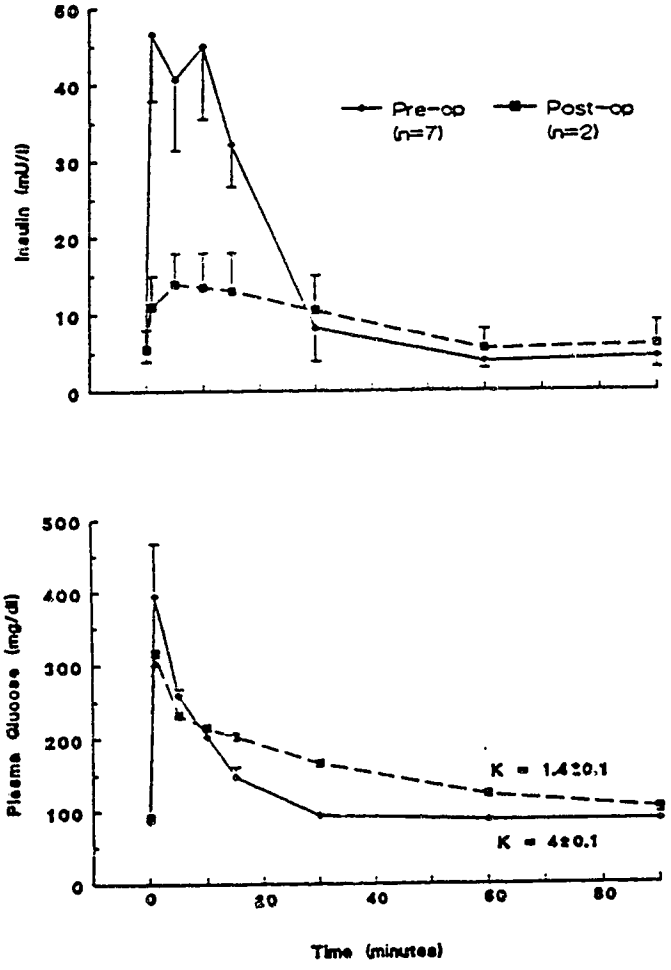

FIGURE 4-3: Plasma concentration (mean±SEM) of glucose and insulin during IVGTT, pre- and postoperatively in recipients of long-surviving cultured allografts.

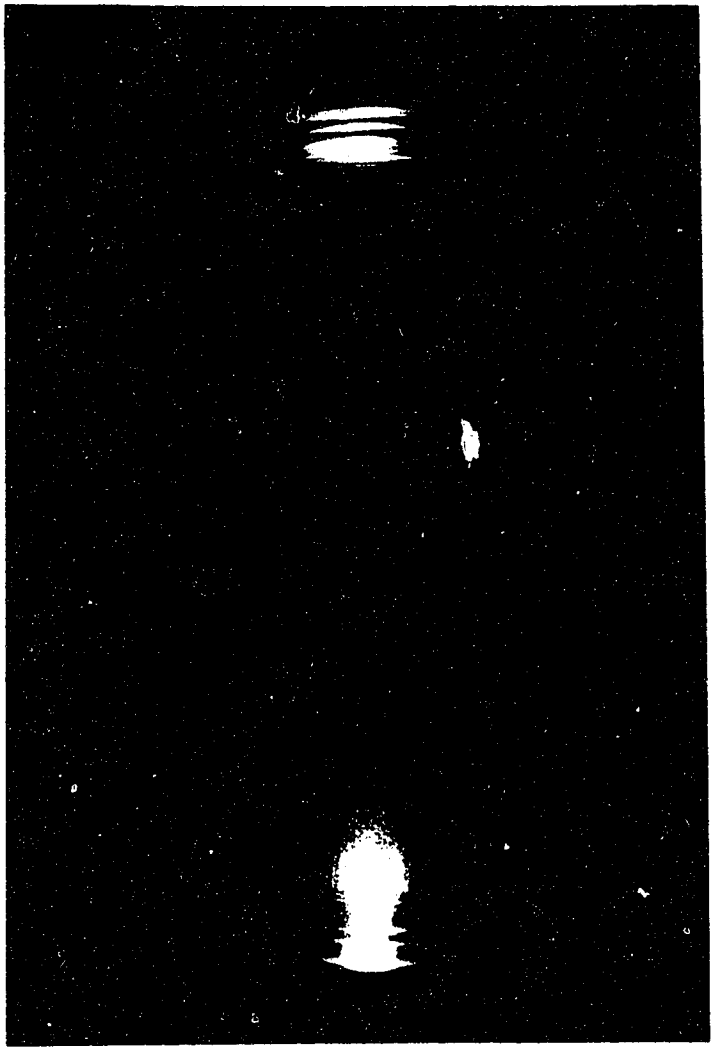

PLATE 4-1: Photograph of cultured islets in petri dish. The islets were swirled to the center of the dish to facilitate culture-medium changes.

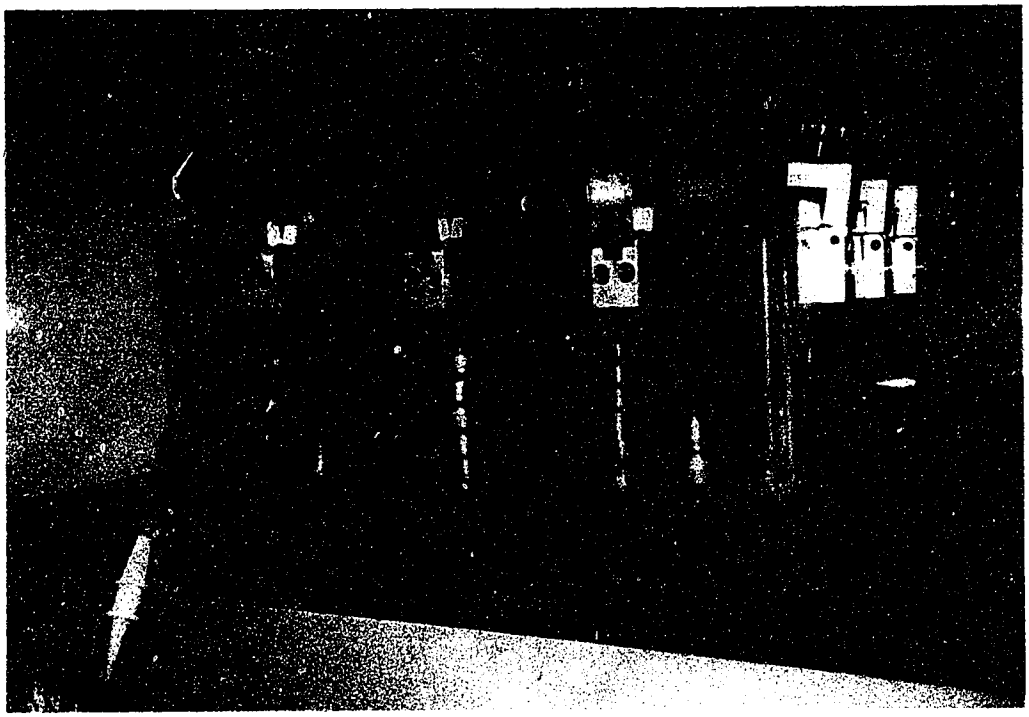

PLATE 4-2: Photograph of the modular incubator used for culturing islets at room temperature.

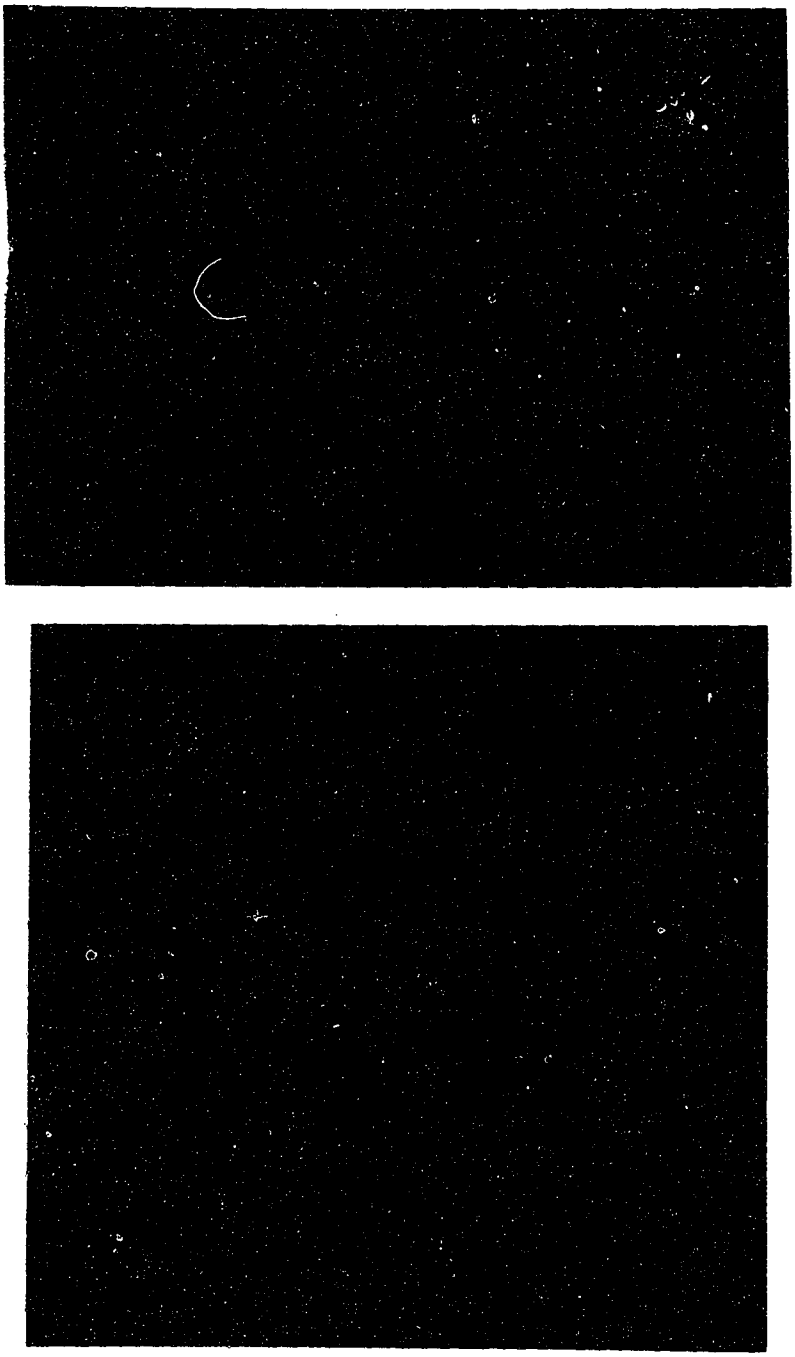

PLATE 4-3: Photomicrograph of purified canine islets after 1 week of culture at 22°C (A) and 37°C (B). Central necrosis increases was frequently seen in large-diameter islets cultured at 37°C.

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CHAPTER 5

DISCUSSION

INTRODUCTION

Our first study demonstrates the feasibility of preventing the rejection of freshly-isolated highly purified canine islet allografts, transplanted into the spleen with CsA immunosuppression. The preoperative CsA serum trough value was important: all the surviving dogs had values >300 μg/L; both of those who had values <300 μg/L rejected their grafts rapidly. While high serum trough values were maintained initially, we were able to taper dosages that provided a mean value of 219±41 μg/L by 4 weeks postimplantation. This compares favorably with the studies of Alejandro et al, who maintained concentrations >500 μg/L (1); this may be attributed to the high degree of purity of the islet grafts in our study (2).

After the cessation of CsA, all grafts promptly failed, which contrasts sharply with the results of studies by Alejandro et al; they reported that purified canine islet allografts implanted intraportally survived at least 30 days after discontinuing CsA - in fact, several grafts survived for many months (3). These authors attributed their success to the high serum CsA levels, which were considerably higher than in our study, and may partly account for the difference between the two sets of data. Our results suggest that continued CsA treatment which provides adequate serum trough values is necessary to ensure continued graft survival.

Although overt manifestations of CsA-induced toxicity were not seen in the present study, the metabolic performance of the allografts in the long-surviving recipients was clearly impaired, which may reflect an adverse effect of CsA on islet engraftment (4)

or insulin release (5-8). In contrast with dogs, humans would not likely tolerate the serum levels of CsA we employed, although it is unlikely that single-agent immunosuppression used in our studies, would be applied clinically. As pancreatic islet transplantation enters the clinical arena, effective, nontoxic antirejection strategies become essential — particularly if it is to be applied to diabetics who do not manifest, but are destined to develop, the secondary vascular complications of the disease. We therefore evaluated two approaches that have been used successfully in experimental models that facilitate islet allograft acceptance without prolonged immunosuppression: first, transplantation of islets beneath the renal capsule, a site reported to be immunologically privileged (9,10); and second, immunoalteration by in vitro islet culture at 22°C for 1 week before transplantation (11,12).

TRANSPLANTATION OF ISLET ALLOGRAFTS BENEATH THE RENAL CAPSULE

The duration of function of islet allografts implanted into the renal subcapsular space was significantly shorter than those implanted into the spleen, findings that are particularly striking in view of the greater quantity of islets provided (20% more than in the splenic site), and serum CsA levels >300 μg/L on the day of transplantation in all recipients. While functional impairment may be attributed to immune processes causing progressive loss of islet mass, it may also reflect impaired islet engraftment beneath the renal capsule - a theory partly supported by histologic evidence of graft survival in 3 of 6 dogs 30 days postimplantation.

It is possible that the microenvironment of the canine renal subcapsular space has intrinsic deficiencies in vascularity or other tropic influences which prevents or limits islet neovascularization. High serum levels of CsA during the first week after transplantation

also may have contributed to islet loss by reducing or redistributing renal blood flow (13). Other factors that have may have adversely affected islet engraftment in the renal subcapsular space include hematoma caused by surgical trauma (17), and the diabetic state which has been shown to impair blood perfusion of islet grafts after transplantation (15,16).

Systemic venous drainage of the renal subcapsular grafts is another factor that may have reduced the duration of normoglycemia: comparison of the function of islet autografts of equal mass with systemic and portal venous drainage showed significantly better function of the latter (17,18). It is possible that reduced efficiency of an initially marginal mass of islets may have overwhelmed the functional capacity of the ß cells, resulting in metabolic deterioration, and ultimately graft failure. In rodents, this problem may be partly overcome by implanting a relatively large quantity of islets: four hundred islets are commonly implanted beneath the renal capsule of mice weighing 25 g (20,000 islets/kg) to induce normoglycemia, a quantity that far exceeds that used in our studies.

While sustained normoglycemia did not occur, marginal graft function (PG <225) was evident in 3 of 6 dogs for almost 14 days. This compares favorably with the studies of Hess et al (20), and Evans et al (21), who reported that autografts of canine pancreatic microfragments implanted beneath the renal capsule uniformly fail immediately after transplantation. Our improved results may be due to the high purity of our islet grafts, which minimizes the potential adverse effect of exocrine secretion on islet engraftment. Nevertheless, our results contrast sharply with those reported by Toledo-Pereyra, in which mechanically prepared pancreatic microfragments induced prolonged normoglycemia, even in nonimmunosuppressed recipients (10). However, no laboratory has been able to reproduce these results, and therefore they should be interpreted cautiously.

The inability of islets implanted beneath the renal capsule to induce prolonged normoglycemia in our study suggests that the practical value of this site is limited in this dogs. Whether an immunologic advantage exists, remains to be determined.

TRANSPLANTATION OF CULTURED ISLET ALLOGRAFTS

One of the major criticisms of in vitro immunoalteration of pancreatic islets is the potential for the loss of islet mass and/or viability. In rodents, this problem is partly resolved by the availability of inbred strains, which permit the use of multiple syngeneic donors to acquire sufficient numbers of islets. However, applying immunoalteration techniques to islets obtained from outbred large animals and humans is contingent upon in vitro conditions that ensure consistent recovery of the critical islet mass necessary to induce recipient normoglycemia.

Our studies demonstrate that the loss of viable islets after 1 week of culture at 22°C is minimal. Moreover, their functional integrity is well maintained as shown by the rapid induction of normoglycemia in all recipients. The results of our in vitro studies suggest the success of our culture conditions may be due to the low temperature used. This concurs with studies of rat and human islets which have shown better preservation of the morphologic and functional integrity of islets cultured at 22°C versus 37°C (22,23). Our ability to recover large quantities of canine islets after culture allowed us to investigate the function and survival of cultured purified islet allografts transplanted from single donors into the spleen of dogs immunosuppressed with CsA at serum levels less than those used for freshly isolated islet allografts (Chapter 2).

Our data indicates that 1 week of in vitro culture may facilitate the acceptance of canine islet allografts, as shown by prolonged graft function in 2 of 5 dogs treated with low serum levels of CsA. The failure of these grafts shortly after the cessation of CsA

contrasts sharply with reports of other studies, in which in vitro culture in combination with brief course of recipient immunosuppression appeared to induce immune unresponsiveness to islet alloantigens (10,11). Lacy et al reported >100 day survival of cultured rat allografts after a single injection of ALS (11). In the study by Terasaki et al, purified rat islet allografts cultured for 1 week at 24°C survived >60 days after three perioperative subcutaneous injections of CsA (11). However, serum CsA levels were not measured in that study; and significant serum levels may persist for a long period of time after large depot injections (24). Our results suggest that continued CsA treatment, although at levels less than those needed for fresh allografts, will be necessary to ensure continued survival of cultured islet allografts.

Early failure of cultured islet allografts in the remaining 3 dogs suggests that the reduction of islet immunogenicity after 1 week of culture may be variable. While this may be due to unrecognized inconsistency of culture conditions, it may also reflect differential susceptibility of islets obtained from a heterogeneous donor pool to immunoalteration. Resolution of this issue, however, must await further clarification of the molecular processes occurring within each of the constitutive cell populations of islets during culture — and only then will rational modification of the culture conditions be possible.

If there is indeed a variable response of islets obtained from different donors to tissue culture, will this approach be feasible clinically? While there is little data that examines this issue directly, preliminary studies by Alejandro et al. show that applying a variety of immuno-alteration strategies in combination or sequentially can consistently effect sufficient reduction of islet immunogenicity in a large animal model (25). Perhaps what is needed most to advance this approach clinically is a reliable in vitro correlate of immunoalteration that is easily measured. In this regard changes of islet MHC antigen

concentration during culture may have practical significance (26,27). However, it remains to be determined if altered antigen expression represents permanent elimination or inactivation of specific cell populations (for example, passenger leukocytes), or just temporary down-regulation of antigen expression.

CONCLUSIONS

1) Canine islet allografts are immunogenic and are rapidly rejected in an untreated recipient.

2) CsA at serum trough values >300 μg/L by RIA will prevent the rejection of highly purified single-donor canine islet allografts.

3) Islet allograft rejection is prompt after the cessation of 30 days treatment with CsA, indicating that this duration of CsA therapy does not induce a state of immune unresponsiveness to islet alloantigens in an outbred large mammal.

4) Metabolic efficiency of canine islet allografts in combination with CsA therapy is impaired compared with autografts.

5) Islet allograft survival is compromised in the renal subcapsular space.

6) The recovery, morphology, and functional integrity of canine islets is well maintained during in vitro tissue culture at 22°C for 1 week.

7) Preliminary studies suggest that low temperature tissue culture may facilitate canine islet allograft acceptance, although further studies are needed to confirm this advantage.

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