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IMMUNE INTERVENTION AND THE DELAY OF THE RECURRENCE OF DIABETES AFTER ISLET TRANSPLANTATION IN THE NOD MOUSE

BY

ANNABELLE J. S. SYDIE

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

IN

EXPERIMENTAL SURGERY

DEPARTMENT OF SURGERY

Edmonton, Alberta

FALL, 1994

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DEGREE: Master of Science

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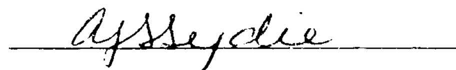

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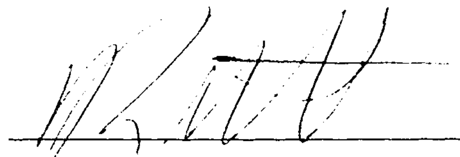

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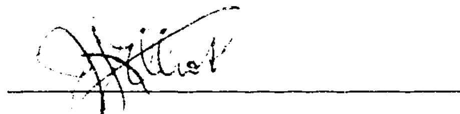

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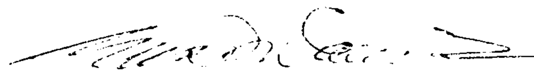

Dr. G. L. Warnock

This text is dedicated to my family
and friends
for their continuous support and
encouragement

ABSTRACT

Islet transplantation, which is now being used with success clinically, faces two major immunological problems, allorejection and autoimmune destruction. Allograft rejection has been prevented by *in vitro* immunomodulation of the islets however, few methods have demonstrated complete elimination of graft invasion by monocytic and lymphocytic cells that destroy the transplanted islet beta-cells. The non-obese diabetic (NOD) mouse is an excellent model to study the pathogenesis of type 1 diabetes. Non-specific immune intervention with microbial derived adjuvants such as bacillus Calmette-Guerin or complete Freund's adjuvant at the time of syngeneic islet transplantation in a diabetic NOD mouse prevents islet beta-cell destruction and disease recurrence. The aim of this study was to isolate the component of these adjuvants that provides the protective effect and to develop a more clinically relevant treatment to prevent autoimmune destruction of the transplanted tissue.

Islets isolated from young NOD mice by collagenase digestion and Ficoll purification were transplanted under the kidney capsule of diabetic NOD recipients. To identify the protective effect of CFA and BCG, heat-killed *Mycobacteria* at selected doses were administered at the time of islet transplantation into a diabetic mouse. As well, two specific autoantigens, glutamic acid decarboxylase and heat shock proteins were studied for their role in preventing disease recurrence post-islet transplantation.

The results from the treatments with dead *Mycobacteria* indicate that for BCG to induce a state of tolerance and thus protect the islets

from autoimmune destruction, the bacteria must be live and administered in a large dose. The protective effect of CFA can be attributed to the mycobacterial components in CFA and can not be attributed to the oil component of this adjuvant. This study found no role for the autoantigen GAD in preventing the disease recurrence of diabetes. The heat shock protein derived from *Mycobacterium leprae* was effective in inducing a state of tolerance to the transplanted islets which were not destroyed by autoimmune destruction.

The immune stimulative property of bacterial adjuvants and heat shock proteins has been documented previously and it is assumed that an interruption in the course of disease through the administration of an immune stimulant suppresses the autoimmune process of beta-cell destruction.

ACKNOWLEDGEMENTS

I wish to express my gratitude:

To my supervisor, Dr. R. V. Rajotte, for the opportunity to work, study and be part of the Islet group. Also for his continual guidance and generous support throughout the years.

To Dr. J. F. Elliott who provided the inspiration for much of the work presented in this text. As well, to D. K. Smith, for his constant assistance and encouragement throughout the my program.

To Dr. G. L. Warnock for his advice, support and encouragement.

To the members of the CORE laboratory, Deb Dixon and Stephanie Martineau, for their continuous patience and assistance throughout my study and mostly of all for their friendship.

To all the members of the Islet laboratory for their technical support and friendship.

To Dawne Colwell and Doug Bracewell for their graphical and technical assistance and especially for their patience.

To Rosemarie Henley and Colleen Gardner for their patience and continual support throughout the years and for helping me complete this thesis.

Financial support provided by the Juvenile Diabetes Foundation International (DIRP) and the Department of Surgery at the University of Alberta.

The never-ending support from my family and my best friend Kelly have allowed me to complete this study with a feeling of accomplishment.

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LIST OF ABBREVIATIONS

ALS	anti-lymphocyte serum
APC	antigen presenting cell
ß-cell	beta-cell
BB	bio-breeding (rat)
BCG	bacillus Calmette-Guerin
CFA	complete Freund's adjuvant
CsA	cyclosporin A
ENCV	encephalomyocarditis virus
GAD	glutamic acid decarboxylase
HBSS	Hank's balanced salt solution
IAA	insulin autoantibodies
ICA	islet cell antibodies
IDDM	insulin-dependent diabetes mellitus
IFA	incomplete Freund's adjuvant
Il	interleukin
IFN	interferon
LCMV	lymphocytic choriomeningitis virus
LDSZ	low-dose streptozotocin (model)
LDV	lactic dehydrogenase virus
MHC	major histocompatibility complex
MHV	mouse hepatitis virus
NOD	non-obese diabetic (mouse)
PFSY-A1	*Plasmodium falciparum* derived control protein
VAF	variable antigen-free

I
INTRODUCTION

Insulin-dependent diabetes mellitus results from the autoimmune destruction of the insulin-producing cells in the pancreas (1). In 1889, Von Mering and Minkowski (2,3) discovered pancreatic diabetes, this being many years after the records in the Papyrus Ebers of ancient Egypt which tell of a wasting disease associated with frequent urination and weight loss (4,5). Until this century, man had not been able to pinpoint the cause of this devastating disease that has claimed many lives. After a long and painful history, diabetes mellitus is now referred to as a metabolic disorder of insulin deficiency or inadequate function (6). Diabetes is characterized by abnormalities in glucose metabolism that result in ketoacidosis, thirst and increased urine production in patients with the disease (7). Prior to 1921 when Banting and Best discovered insulin, this disorder was accompanied by death within a year of diagnosis (8). Along with the disease itself, which can be controlled with exogenous insulin administration, the late stage complications of diabetes include retinopathy eventually leading to blindness, cardiovascular disease, nephropathy causing renal failure and neuropathic syndromes (9-15).

For the nearly eleven million North Americans afflicted with diabetes, many of the clinical complications of the disease itself interact with other risk factors. These include arteriosclerosis which is ten times more common in a diabetic than the general population, and myocardial infarction which is doubled (15). Diabetic

retinopathy is one of the leading causes of blindness in North America (16) and the United Kingdom (17). Death due to renal disease is 23 times more common in type 1 diabetic patients compared with the general population (15). Experimental and clinical studies have shown that these complications are the result of the metabolic disturbances caused by diabetes and the extent of hyperglycaemic periods. For example, Engerman *et al* noted in 1977 that pancreatized dogs receiving a regimented program of two daily injections of insulin for 60 months showed fewer retinal lesions than dogs that did not receive insulin injections on a regular basis (18).

It is now recommended that therapy for a diabetic patient include the strictest possible control of the blood sugar level through insulin delivery and administration of oral hypoglycaemic agents (19,20). Daily insulin injections cannot control the wide swings in blood glucose unless given in multiple doses or by an insulin pump system, both requiring a high degree of self-monitoring by the patient. Many agree with the theory that exogenous insulin is a treatment for diabetes not a cure (4).

Diabetes research is an essential part of the quest for a cure for this disease that inflicts so many lives. There are many aspects to current research on diabetes mellitus including clinical studies in pancreas and islet transplantation as well as genetic and immunological studies on the pathogenesis of the disease. Many clinical studies are based on improving the efficiency of metabolic management of diabetes and the reversal or prevention of diabetic complications (19,21). Research into the genetics of diabetes is

concerned with the prevalency of disease in certain individuals with certain genotypes and the higher frequency of HLA alleles that occur in patients with diabetes as compared to the general population (22). Attempts to understand the immune mechanisms of pancreatic ß-cell destruction in type 1 diabetes is a key part of immunological research in diabetes (23,24). Clinical application of transplantation of the insulin producing tissue into a diabetic patient is a reality and many centres around the world are concentrating their efforts on the optimization of pancreas and/or islet cell transplantation (25-28).

IMMUNOGENETICS OF IDDM

Diabetes mellitus has been considered an inherited disease for nearly 3000 years. Many historical accounts of the disease have a recurrent theme of the familial tendency of this disorder (4). The exact mode of inheritance of the susceptibility to diabetes has not been established although much attention has been focused on the association of insulin-independent diabetes mellitus (IDDM) with specific alleles in the MHC locus (22). It has been determined that IDDM is associated with HLA-DR3 and HLA-DR4 on chromosome 6 and with the presence of a non-charged amino acid at position 57 of the HLA-DQ beta chain, one of two structures making up the HLA heterodimer (22). Class II gene products control cellular interactions involved in immune responses and because of this role it can be assumed that MHC gene products are involved in autoimmune processes (29). The association of class II MHC genes with IDDM indicates a direct role in autoimmune reactions (1,29-30).

Diabetes mellitus is an autoimmune disorder (1,31,32). The body's own immune system destroys the cells in the pancreas that produce insulin (ie. the ß-cells). To put it another way, immunological tolerance for a component of self, the pancreatic ß-cells, is lost or perhaps never achieved in the first place through a fault in negative selection in the thymus (32). Through a complex system of genetic and environmental triggers, the immune system integrates both humoral and cellular forces that lead to the destruction of ß-cells (24,33). The immune system was first implicated in the development of IDDM when the disease's associations with other autoimmune diseases and the morphological evidence of mononuclear infiltration in the pancreas first came to light (30). Other evidence showed that the serum of newly diagnosed Type 1 diabetics almost always contained islet cell antibodies (ICA) (34,35), and the predisposition of persons with certain HLA-DR and DQ genotypes to develop IDDM (22) served to implicate the immune system further in the disease process.

Diabetes has a higher association with other autoimmune diseases (usually affecting other endocrine organs) than would be expected by chance, and it can sometimes be part of a syndrome of polyendocrine diseases (23,24,36). The most common of these disorders in IDDM patients is autoimmune thyroid disease, which occurs approximately 30 times more often in patients with diabetes than in the general population (36). The pathological element linking these disorders remains unknown (30,36).

Early morphological studies of persons that have died close to diagnosis of diabetes revealed insulitis that is an inflammatory infiltrate of lymphocytic cells in the islets of Langerhans (23,37). The ß-cell mass comprises only 1 to 2% of the volume of the largely exocrine pancreas and this mass can be reduced to one-tenth of the original amount at the onset of IDDM (37). The destruction of the ß-cells accounts for the severe insulin deficiency, while the remaining endocrine cells of the pancreas, the alpha and delta cells, continue to maintain their normal physiological secretory function and are not subject to damage from the immune system (37). The majority of lymphocytes infiltrating the ß-cells are of the cytotoxic/suppressor phenotype cells, indicating that the pathogenesis of IDDM may be a cell-mediated process (32,38-41). Evidence from pancreatic transplantation between identical twins shows that autoimmunity is responsible for the recurrence of IDDM in these patients (42). Reports of an accelerated re-enactment of the pathogenic sequence of ß-cell destruction following twin to twin pancreas transplantation clearly illustrates the autoimmune nature of type 1 diabetes.

Islet cell antibodies (ICA) and anti-insulin autoantibodies (IAA) are now common serologic markers for ß-cell autoimmunity, and are being used to predict the development of type 1 diabetes (34,35). The presence of these circulating antibodies in prediabetic patients is additional evidence that ß-cell destruction is immune mediated (31). Insulin autoantibodies are present prior to the exogenous administration of insulin, and therefore it has been suggested that insulin produced by the ß-cells may serve as a surface and secreted

antigen which stimulates the immune system (31,32). A third autoantibody (64kD autoantibody) has also been discovered which reacts with a native 64kDa islet protein. This islet protein has been recently identified as the enzyme glutamic acid decarboxylase (GAD) (43,44).

INTERVENTION STRATEGIES

Presently, novel therapies to prevent or halt further ß-cell destruction once the serum markers are discovered in a patient's blood are being tested at centres around the world (44). These treatments attempt to directly target or prevent the autoimmune attack of the islet ß-cells. Two strategies of immune intervention for clinical application have evolved because of successes in the rodent models of IDDM. The first of these is to identify those at high risk for the development of diabetes and to intervene with immunomodulation well before disease onset. The second approach is to halt ß-cell destruction once it has already begun in those patients that were not originally identified as at risk for the development of diabetes (31,44). In those who are first degree relatives of patients with IDDM, HLA phenotyping and ICA detection allow reasonable prediction of IDDM risk (44). Unfortunately, only a minority (10%) of newly diagnosed diabetics have a first degree relative with diabetes and therefore a large scale screening method to assess risk for IDDM in the general population will be necessary if immune intervention in diabetes is to become widespread (44,45).

Cyclosporin A (CsA) is a non-cytotoxic immunosuppressive agent which is selectively directed against helper T-lymphocytes (46). Initial clinical trials with newly diagnosed diabetic patients receiving CsA treatment were aimed at preventing further destruction of the pancreatic ß-cells by autoreactive T-lymphocytes (31,44-47). Unfortunately treatment with CsA resulted in a high incidence of acute nephrotoxicity in the study groups, with only a few patients exhibiting insulin free remissions for periods of up to one year (44,47-49). Subsequent CsA study groups have demonstrated that CsA does not have a lasting preventative effect on ß-cell destruction, and that this destruction is resumed once treatment is discontinued (44). As always, the general risks of immunosuppression, plus the potential nephrotoxicity with continued use of CsA must be weighed against the benefits of immune intervention (44-49).

Nicotinamide, a water soluble B vitamin, has been reported to reduce ß-cell damage and prevent development of clinical diabetes in animal models (50,51). The most promising results to date using nicotinamide have been from its use in uncontrolled studies with prediabetic subjects (44,52). Nicotinamide has the advantage of being relatively safe, unlike the powerful immunosuppressive drug cyclosporin described earlier (44). Centres around the world are collaborating an ongoing trial to evaluate the effect of nicotinamide in prediabetic high risk subjects, those with a relative with diabetes and circulating ICA in their blood (44,53).

In another major study in the United States, insulin is being given as a preventative therapy (44). The rationale for using insulin

therapy to prevent or arrest the progression of ß-cell damage is that by introducing extra exogenous insulin to the immune system, this may reduce the antigen stimulus associated with endogenous insulin secretion (54,55). This serves as an immune modulator decreasing the ß-cells susceptibility to immune attack. Unlike other intervention studies discussed, insulin has the benefit of having a well documented history in diabetes, and can be easily controlled for side-effects (44). The results from a study initiated by the Joslin group indicated that the use of insulin therapy at the late prediabetic stage may be effective in producing ß-cell rest and the prevention of overt clinical diabetes (56,57). Additional trials throughout the world are presently ongoing to fully evaluate the beneficial effects of insulin (44).

Novel therapies such as treatments with anti-T-lymphocyte preparations (58) or localized pancreatic irradiation (59) are being evaluated with controlled clinical trial, having been already evaluated in small preliminary study groups where preserved islet function was demonstrated in control subjects.

These reports and trials indicate that the natural history of IDDM can be altered by immune intervention in new onset diabetic patients. It is likely though that these strategies of intervention were administered too late in the clinical course of the disease, as the results indicate a prolongation of the honeymoon period, but not prevention or reversal of the disease (44,47-49,60,61) It has been suggested that intervention programs should begin in prediabetics, when there is a larger ß-cell mass still present and therefore increasing the likelihood of preventing the clinical expression of

IDDM (44,60,61). Although these patients are asymptomatic, there is the risk of progression to overt diabetes, and until an accurate indicator of this progression is discovered, it is not likely that prediabetic subjects will be used to test new therapies unless they are relatively harmless, as is the case with nicotinamide (26,44,60-63).

PANCREAS TRANSPLANTATION

The theory of transplantation of insulin producing tissue has long been accepted as a better treatment for IDDM than exogenous insulin therapy. Williams and Harvant (64) were the first to attempt a pancreatic transplant on a human when they treated a young boy with subcutaneous implants of pieces of freshly slaughtered sheep pancreas. Three days later the boy became comatose and died. Histology of the graft showed only fibrous stroma. This report precedes that of Ssobolew (65) who is said to have been the first to suggest transplantation of islets as a treatment for diabetes that could possibly slow down the development of its complications. Unfortunately many early transplants met with little success, and researchers tended to move on to the more promising studies of that period. With the discovery of insulin by Banting and Best at the University of Toronto (8), the focus on transplanting pancreatic tissue entered a dormant phase while medical therap · ,oared ahead (4).

Eventually pancreatic transplantation was recognized as the most therapeutic and physiological treatment for diabetes mellitus. This was partly due to the fact that insulin therapy had its downfalls, namely that the late complications associated with diabetes mellitus

still occurred (19,20). Another barrier to insulin therapy is that patients with type 1 diabetes are at constant risk for severe hypoglycaemia due to the pharmacologic administration of insulin (7,15). The therapeutic approach using insulin is unable to alter significantly the morbidity and mortality caused by the vascular disease seen in diabetic patients (7,15). Since the primary defect is in the ß-cell, it was hypothesized that successful transplantation of normal ß-cells into a diabetic patient could not only normalize blood sugar, but might prevent the onset of vascular complications in the brain, eye, kidney, peripheral nervous system and other tissues (66).

Since 1966, transplantation of the vascularized pancreas has become a common procedure both in clinical and experimental settings (67-71). Von Mering and Minkowski (2,3) sparked interest in the possibility of transplanting pancreatic tissue when they reported the onset of hyperglycaemia after the removal of the pancreas in experimental dogs. A few of the problems encountered by researchers in the early years of pancreas transplantation involved the auto-digestion of tissue by the digestive enzymes secreted by the exocrine portion of the pancreas, and rejection of the transplanted pancreas by the host (72,73). These problems, especially the former, prompted researchers to isolate the pure form of the insulin secreting tissue for transplant, the islets of Langerhans.

Up to 4000 pancreas transplants have been performed since 1966, and this figure continues to grow as the procedure becomes a frequently used therapy for selected IDDM patients undergoing simultaneous kidney transplants due to end-stage renal disease (68-

70). Improvements over the past decade have led to a 1-year graft survival rate (insulin-independence) in 75% of patients receiving simultaneous pancreas/kidney transplants (69). The additional surgical risk of pancreas transplantation has been justified by the fact that these patients are already obligated to take immunosuppressive agents such as cyclosporin, azathioprine, and prednisone because of the combined kidney transplant (67,69). While the immediate benefits of this procedure when successful are improved glucose metabolism and the elimination of exogenous insulin administration, the long-term beneficial effects on the development and severity of vascular complications has not been completely evaluated. Preliminary results indicate that improvement of nephropathic and neuropathic syndromes does occur with prolonged acceptance of whole pancreas grafts, whereas progression of retinopathy still occurs (69,70,74,75).

PANCREATIC ISLET TRANSPLANTATION

Whole pancreas transplantation has proven to be moderately successful clinically yet it is still a difficult and relatively costly procedure when undertaken without a simultaneous kidney transplant. Other limitations to this procedure include the requirement for major surgery, continuous immunosuppression, and organ donor shortage all of which combine to make it an unlikely strategy for the long term treatment of IDDM (26,27,71,75). Pancreatic islet transplantation offers possible advantages over pancreas transplantation for several reasons: 1) it is a minor as opposed to a major surgical procedure, 2)

islets can potentially be altered to eliminate or reduce the requirement for post-transplant immunosuppression (25-28,76), 3) long-term preservation of the pancreatic islets has been made possible by cryopreservation of the purified tissue. Transplanting endocrine cells free of exocrine tissue also reduces the risk of complications such as thrombosis of the whole pancreas graft (25-28,76). Insulin independence in patients receiving islet grafts has been achieved for variable lengths of time in many centres (25,76) with the best success to date being that in Edmonton with two of the patients going long term without the need for insulin (25).

The successful isolation of islets as free tissue came in 1965 when Moskalewski isolated intact islets from a guinea pig pancreas incubated in an enzyme complex derived from *Clostridium histolyticum* (77). The technique of isolating intact islets using collagenase was subject to further research and a modified isolation procedure was reported by Lacy in 1967 (78). By mechanically disrupting rat pancreases by a ductal injection of salt solution prior to collagenase digestion, a higher yield of islets was obtained. The isolation procedure also included the use of a discontinuous sucrose density gradient that effectively separated islets from acinar debris. The sucrose density gradient was later replaced with a Ficoll gradient by Lindal *et al* who since this sucrose polymer provided a better osmotic environment for the islets (79). In 1973, Scharp *et al* reported the use of lyophilized and dialyzed Ficoll which removed impurities that may have effected islet viability (80). Islets purified with dialyzed Ficoll showed normal function *in vitro* as compared to

islets purified with undialyzed Ficoll, where insulin secretory function was impaired following a glucose challenge. The use of reflected green light to identify isolated islets and discriminate them from small lymph nodes *in vitro* was developed by Finke *et al* and enabled easy handpicking of purified islets for experimental transplantation (81).

Younoszai and associates reported in 1970 (82) the first study where transplanted rodent islets were successful in inducing a short-term euglycaemic state in chemically induced diabetic rats. Ballinger and Lacy (83) isolated rat islets using the collagenase digestion and Ficoll purification in 1972 and subsequently were able to partially reverse experimentally induced diabetes by transplanting these islets into the peritoneal cavity of diabetic rats (83). It was not long after this report of moderately successful islet transplantation that Kemp et al demonstrated that islets transplanted into the liver via the portal vein could completely normalize diabetic rats. (84). The first study reporting the transplantation of pancreatic tissue under the renal subcapsular space was in 1976 (85). This report demonstrated that placement of fetal pancreases beneath the kidney capsule of syngeneic diabetic rats resulted in complete reversal of the diabetic state. Until this time, transplantation of islets in the rat model were primarily via the portal vein technique as the liver seemed to offer the most physiological site for islet transplantation (86). The subcapsular space was chosen because of rapid vascularization and accessability for islet transplantation (85).

Using the isolation techniques described by Moskalewski, Lacy and Lindal, researchers attempting to isolate murine pancreatic islets typically obtained low yields of islets, usually less than 100 per animal (87). Successful transplantation to reverse streptozotocin-induced diabetes therefore required several donor pancreases. Gotoh et al in 1985 reported using a stationary digestion technique for the isolation of islets in mice which resulted in more than 200 viable islets from each pancreas (88). As well, intraductal injection of collagenase enhanced the separation of islets from exocrine tissue (88). The renal subcapsular space was reported in 1986 to offer better growth conditions for transplanted mouse islets when compared to the intrasplenic and intraportal sites of islet transplantation (89). Gores in 1987 reported that the portal vein provided a privileged site for murine islet allografts when compared to the renal site (90). The difference in results between the two previously mentioned studies, the first having transplanted syngeneic islets and the second allogeneic islets, led researchers to speculate that the better vascular conditions of the renal site contributed to higher rates of rejection when islet allografts were transplanted to this site (91).

As animal models of IDDM became available, the possibility of studying the pathogenesis of autoimmune destruction of transplanted islet tissue *in vivo* became a reality. Persistence of autoimmune disease after initial ß-cell destruction in the pancreas was evident in humans, where the recurrence of the autoimmune disease process occurred rapidly in pancreases transplanted between discordant twins (41), and in NOD mice, where the destruction of syngeneic pancreatic

ß-cells occurred rapidly in islets transplanted into diabetic recipient mice (92-94).

RODENT MODELS OF IDDM

Observations on the pathogenesis of human IDDM are difficult to obtain for practical and ethical reasons. In order to extend our knowledge of IDDM-like syndromes and of insulin producing tissues in transplantation, investigators have found animal models that share many features with human IDDM. The small animal models used to elucidate the etiology of IDDM are: 1) the low-dose streptozotocin model (LDSZ) (95), 2) the bio-breeding rat (BB) (96), and 3) the non-obese diabetic mouse (NOD) (97). The streptozotocin model represents the induction of diabetes by a specific ß-cell toxin, and the last two provide models for spontaneous onset IDDM.

Low-dose streptozotocin provides an experimentally induced animal model of IDDM (95). Multiple injections of low doses of streptozotocin (5 x 40 mg/kg), a ß-cell toxin, produces diabetes by the destruction of the pancreatic islets although the destruction of the islets is not as complete as when administered in a single high dose (200 mg/kg). Following low-dose streptozotocin injections, intense insulitis is induced, and this inflammatory process appears to be responsible for the destruction of the ß-cells (95,98,99). A genetic predisposition to LDSZ induced diabetes is demonstrated by the fact that female mice are partially resistant to the induction of diabetes (98). The immune nature of IDDM in this model has been proven with experiments which show that LDSZ diabetes cannot be induced

in T-cell deficient mice, a characteristic which is reversed with thymus grafting (98) in order to transfer the disease with splenocytes. Furthermore, T-cells must be present (98). Although the precise etiological mechanisms for diabetes in the LDSZ model are not known, researchers have postulated that streptozotocin produces an initial nonspecific islet inflammation (98). This initial insult results in killing of inflamed ß-cells by macrophages, as well as direct toxic effect of the drug. A recent study by Elias *et al* concluded that the autoimmune diabetes that results from a LDSZ in BK (C57BL/KsJ) mice was immunologically similar to the autoimmune diabetes which occurs in NOD mice (99). This conclusion was based on the fact that similar autoantibodies to insulin were detected in both strains of mice in the prediabetic stage. The significance of these findings is that the mechanism of ß-cell destruction by the LDSZ model has a different trigger than the spontaneous disease progression in the NOD. The end result of both is autoimmune diabetes indicating that the immune system can be triggered to produce an autoimmune response by different environmental factors (98,99).

The BB rat and the NOD mouse are models of spontaneous diabetes that resemble human IDDM. The BB rat was discovered two decades ago in a colony of outbred Wister rats at the BioBreeding Laboratories in Ottawa, Canada (96). The NOD mouse, developed by Makino (97), will be discussed in greater length in the next section. Spontaneous diabetes appears in BB rats between 60 and 120 days of age (96). Mononuclear infiltration of the pancreatic ß-cells by autoreactive T lymphocytes starts 2 to 3 weeks before the onset of

overt diabetes (100). Immunological studies of the insulitis that develops in the BB rat indicate that this infiltrate is mediated by an autoimmune process (101). Inheritance of diabetes in BB rats appears to be closely linked with the class II region of the MHC. Unlike the NOD mouse, the BB rat has a similar incidence of diabetes among both the males and females, whereas female NOD mice have a substantially higher incidence of diabetes than do the males in the same colony (101). The BB rat has proven to be a useful model for therapeutic trials in IDDM. Immune intervention during the prediabetic stage in BB rats has been successful in preventing the onset of disease, for example by the administration of cyclosporin (102), CFA (103), anti-lymphocyte serum (104), or monoclonal antibodies directed against class II MHC antigens (105). Insulin treatments given to young BB rats have also been shown to inhibit the development of diabetes as well as reducing the mononuclear leukocyte infiltrate into ß-cells (106). The prevention of diabetes by immune intervention is a strong argument for the autoimmune nature of diabetes in the BB rat.

THE NON-OBESE DIABETIC MOUSE

The NOD mouse is an inbred strain developed by Makino from non-inbred ICR mice (97). The resultant diabetes in the NOD is caused by a T-cell dependent pancreatic infiltrate which selectively destroying the insulin producing ß-cells. Between 4 and 12 weeks of age, NOD mice of both sexes develop an increasingly severe pancreatic infiltration consisting largely of macrophages and

lymphocytes (both $CD4^+$ and $CD8^+$ subsets) (107-111). Eventually many of these animals (especially the females) manifest a clinical syndrome of insulin deficiency, hyperglycaemia, ketosis and overt diabetes (97).

In most NOD colonies around the world, mice of both sexes show evidence of perivascular mononuclear cell infiltration surrounding the ß-cells in the pancreas beginning at 4 to 6 weeks of age (110). By approximately 12 weeks, almost all animals will have extensive perivascular infiltration and the signs of islet invasion by lymphocytes. Once a critical mass of ß-cells have been destroyed by these autoimmune T-cells, the disease progresses to overt hyperglycaemia (110). According to a recent study by Pozzillo I (112), statistics from the 22 centres surveyed showed that the onset of diabetes in female NOD mice occurs at the median age of 11.5 weeks whereas in males, diabetes occurs at a median of 14.0 weeks. Peak incidence of diabetes is also reported to be much earlier for female mice at 17 weeks as compared to males at 24 weeks. Generally, the overall incidence of diabetes by 30 weeks is much higher in female members of the colony than in males. This pronounced female gender bias for disease is not observed in the BB rat or in humans. The reason for this increased susceptibility to diabetes in female NOD mice is unclear, but probably related to a difference in the immunological response to exogenous stimuli between the males and females in the colony, perhaps regulated by gonadal sex steroids (113). The cumulative incidence of diabetes as reported by Pozzillo at 30 weeks for female mice ranged from 20% to 100% although most

centres reported incidence of diabetes at approximately 80% for the female members of the colony (112).

IMMUNE INTERVENTION IN THE NOD MOUSE

The natural history of the development of IDDM in the NOD mouse is quite predictable due to the inheritance of a gender specific set of susceptibility genes (114). Intervention studies in NOD mice can be easily accommodated to occur at a certain stage of the progression of the disease, for example the presymptomatic stage (up to 3 weeks of age) or the insulitis stage (4 to 8 weeks of age) (111). This useful characteristic of the NOD mouse represents a major distinction of the natural progression of human IDDM versus the animal model of IDDM. The genetic heterogeneity of humans is such that the onset of IDDM can occur anytime after birth to up to 50 years of age, which is contrasted by the predictability of diabetes occurrence in the NOD mouse (113). It can be presumed that there is a prediabetic phase of human IDDM, although the exact pattern and time course of functional ß-cell loss is still unresolved. The possibility of identifying individuals predisposed to IDDM and offering treatment to prevent the onset of disease is the goal of many investigators.

In the last decade, most accounts of the suppression of diabetes in the NOD mouse have reported using either immunosuppressive or immunostimulative agents to prevent disease onset (113,115-118). Immunosuppressive therapy is cytotoxic by nature and immunostimulants are agents that act by enhancing the immune

response which can simultaneously induce suppressor activity (48,119). Researchers use immunostimulants to interrupt or modulate the pathway that mediates ß-cell destruction in order to prevent the disease onset.

Immunosuppressive agents or non-selective drugs such as CsA and FK506, administered to young NOD mice early in the prediabetic phase profoundly reduces the proportion of animals that manifest diabetes (120,121). CsA and FK506 selectively act on both cytotoxic and helper T-lymphocytes which serve to suppress disease onset with the continual administration of the drug. Administration of cyclosporin and FK506 can result in nephrotoxicity, therefore other immune intervention strategies should be explored before the use of these drugs becomes widespread for the suppression of human IDDM. In the case of CsA and FK506, long-term over-immunosuppression with these drugs poses more of a problem than insulin therapy therefore the answer may be in more specific therapy targeting the immune mechanism of diabetes.

Microbial agents and chemicals in the environment have been implicated as triggers of an autoimmune response to ß-cells in genetically predisposed animal models of IDDM. NOD mice and BB rats raised in viral antigen free (VAF) conditions have increased incidence of diabetes as compared to colonies housed in conventional conditions (102,112). As reported by Wilberz, a NOD colony inadvertently infected with the murine hepatitis virus (MHV) had significantly decreased incidence of spontaneous diabetes (122). The suppression of diabetes incidence was suggested to be a result of a

MHV involved immunomodulation or a virus induced inactivation of potentially autoimmune reactive lymphocytes (122). Environmental differences between colonies as reported by Pozzilli who surveyed 21 centres housing NOD mice, may also contribute to the modulation of the immunological mechanisms leading to ß-cell destruction and ultimately reduce the incidence of diabetes (112). Direct infection of NOD mice or BB rats at birth or in adulthood (6 weeks of age) with a variant of the lymphocytic choriomeningitis virus (LCMV), an infection which persists through the animals lifespan, resulted in a significantly decreased incidence of diabetes in the tested animals (123,124). Adoptive transfer of lymphocytes from LCMV infected animals to uninfected naive NOD recipients prevented IDDM in all infused recipients. The virus did not become evident in the recipient mice indicating that the prevention of IDDM was likely caused by virus induced inactivation of autoreactive lymphocytes that mediate ß-cell destruction (123,124). In addition to these viruses, encephalomyocarditis virus (EMCV) (125) and lactic dehydrogenase virus (LDV) (126) infections have been reported to prevent diabetes development in NOD mice.

Immunoadjuvants are compounds whose biological properties give them the capability to activate one of the two CD4, helper T-cell subpopulation, Th1 or Th2, which control major features of specific immune responses (119,127,128). The immunoadjuvants used today for the treatment of several cancers and recurrent infections are microbial derived substances whose bacterial structures provide the major source of these adjuvants (118,127,128). One commonly used

microbial derived adjuvant is Bacillus Calmette-Guerin (BCG) which has been used in immunological experiments and in tumour immunotherapy (129). Experimentally, a single inoculation of BCG in the NOD mouse results in the suppression of insulitis and overt diabetes (130). Another microbial derived adjuvant, CFA also decreases the incidence of diabetes in NOD mice (92,131,132). A single injection of CFA into prediabetic NOD mice significantly reduced the proportion of mice developing diabetes as compared with controls not receiving CFA. In a study by Qin *et al*, young NOD animals receiving spleen cells from protected CFA treated mice were also protected from the spontaneous disease (133). It is hypothesized that the transfer of spleen cell populations from protected CFA animals served to inhibit the generation of effector cells responsible for ß-cell destruction, thus decreasing the incidence of diabetes These reports indicate that non-specific stimulation of the immune system in the prediabetic NOD mouse decreases the incidence of diabetes. Unlike the effect of immunosuppressive agents such as CsA which serve to delete a population of cells, CFA and BCG prevent the onset of disease without the direct elimination of immune cells (120,130-132). It seems contradictory that a powerful stimulant of immune response has such a profound effect on the prevention of IDDM when compared to the well documented effects of agents that suppress the immune system in order to prevent autoimmune disease.

The bacterial components of the immunoadjuvants BCG and CFA differ; CFA is a conventional immune adjuvant preparation of cell wall components *Mycobacteria tuberculosis* and oil, BCG vaccine

contains live *Mycobacteria bovis*. The ability of dead Mycobacteria in water-and-oil emulsions to enhance humoral and cell mediated responses has been noted for decades in laboratory animals and even in cases of human cancer (134-136). CFA, a strong activator of the Th1 cell type, is unacceptable for clinical use as is its counterpart incomplete Freund's adjuvant (incomplete Freund's adjuvant (IFA)), made up solely of oil (136). BCG, as mentioned previously, is approved for clinical use in tumour therapy (129).

The major immune response to intracellular microbial bacteria is cell mediated immunity (137,138). This form of immunity can be adoptively transferred to naive animals with lymphoid cells but not with serum from infected or immunized animals (139). The specificity of cell mediated immunity is due to T-lymphocytes but the effector function for bacterial elimination is mediated by macrophages activated by T-cell derived cytokines (137-139). Protein antigens of intracellular bacteria stimulate strong T cell responses. More specifically, cell wall constituents of *Mycobacteria* activate macrophages which leads to enhanced non-specific resistance and function as adjuvants (134-137). Researchers have attempted to pinpoint the cell wall component of the *Mycobacterium* present in CFA that provides a protective effect. Muramyl dipeptides have recently been refined and are theorized to be a good candidate for the substitute of *Mycobacteria* in CFA and are presently being studied for immunotherapy and adjuvant use (119).

The precise mechanism of protection against the autoimmune disease in mice immunized with a single injection of CFA or BCG is

not known. It is evident that the immune system of the NOD mouse is sensitive enough that the pathogenesis of diabetes can be easily modulated through changes in the functions of the antigen presenting cells (APC) (116,140). Autoimmune disease in the NOD mouse may be a result of changes in the function of the APC contributing to the inability of the immune system to mediate intrathymic deletion of ß-cell autoreactive T-cells in naive NOD mice. Cells serving as accessory cells presenting antigens (APC) to helper T-lymphocytes include macrophages, dendritic cells and B-lymphocytes (139). APC are required to display fragments of foreign protein antigen on their surfaces to be recognizable by T-cells and they also function in the shaping and selection of the T-cell repertoire (139,140). Leiter theorizes that genetic defects predisposing NOD mice to diabetes susceptibility are reflected as maturational or functional defects in the macrophage population (116,140). This theory provides evidence for the resistance to disease susceptibility that occurs when prediabetic mice are exposed to any environmental or immunoregulatory agent expected to activate macrophage function and therefore stimulate antigen processing and presentation. Environmental influences can also intervene with APC function by decreasing the penetrance of diabetes susceptibility genes and upregulating APC function, particularly macrophages (140). This is evidenced by the decreased incidence of diabetes exhibited in NOD females fed with semi-purified diet (141) or exposed to persistent infections (MHV) (122). The prevention of diabetes by direct presentation of the antigen (syngeneic islets) to the thymus during T-cell maturation in young

NOD mice substantiates the hypothesis that the release of autoreactive T-cells from the thymus is a result of abnormality in the thymic environment (defective APC?) (142). Another theory that expands on the upregulation of the APC function which in turn should arrest development of autoreactive cytotoxic T-lymphocytes, is that adjuvant therapy may induce several different cell populations with suppressor activity, natural suppressor cells, macrophages, and T-cells (117,118,143,144). These cells may then serve to inactivate the autoreactive T-cells initially programmed to respond to ß-cell antigen(s) leading to ß-cell destruction and IDDM. Specifically, Rabinovitch *et al* suggest that activation of a Th2 subset of T-cells that produce Il-4 and Il-10 cytokines act in the immune system to down regulate Th1 cell mediated autoimmune response (117,118). Therefore, by stimulating the immunoregulatory mechanisms in the host with therapeutic intervention, it is possible to prevent or circumvent the ß-cell destruction and IDDM.

Shehadeh *et al* conclude that adjuvant therapy with CFA and BCG diverts the immune system from a destructive to a protective effect on the pancreatic ß-cells (145,146). In their opinion, neither CFA nor BCG arrested the autoimmune disease, as evidenced by the aggregation not infiltration of mononuclear cells surrounding the islets of the pancreas. Instead, the immune response to the ß-cells is channelled away from ß-cell destruction. Several recent reports have presented a more precise strategy of targeting the autoimmune responses provoked by ß-cell autoantigens such as glutamic acid decarboxylase (GAD) (43,147,148). Antibodies directed against this

protein are present in prediabetic and newly diagnosed diabetic patients as well as in the prediabetic NOD mouse (43,149-152). The localization of this protein in the ß-cells early in the disease process seemed to implicate this antigen in the initiation of the T-cell mediated ß-cell destruction. Kaufman *et al* reported that a Th1 response to GAD occurs concurrently with the onset of insulitis in NOD mice (147). Insulitis and diabetes were shown to be circumvented by the tolerization of GAD reactive T-cells which in turn blocked the development of T-cell autoimmunity to other ß-cell antigens. It is suggested that the suppression of GAD responsive Th1 cells is responsible for the tolerance evidenced by the decreased production of IFN gamma in antigen stimulated spleen cell cultures from GAD65 tolerant mice (147). Once again it is possible that GAD65 may activate CD4+ Th2 cells, the helper cells that primarily mediate humoral immune responses, which in turn secrete cytokines that suppress the activity of Th1 cells as seen by the decrease in IFN gamma secretion of Th1 type T-cells (147). Tisch *et al* tolerized young NOD mice with GAD65 by intrathymic injections which may have resulted in the negative selection of GAD reactive T-cells inside the thymus thus preventing insulitis and destruction of the ß-cells (148).

Immune responses against a 65kDa mycobacterial heat shock protein have been implicated in the pathogenesis of IDDM as reported by Elias and Cohen (153,154). They have studied the effect of autoimmunity to a heat shock protein, specifically the 65kDa heat shock protein (hsp) of *Mycobacteria tuberculosis* (153,154). Their

studies have shown that autoreactive T-cells responsible for ß-cell destruction recognized and responded to a peptide eptitope within the sequence of hsp65. Administration of the target epitope or the T-cells responding to this epitope to young mice turn off destruction of the ß-cells early in the course of disease (153,154). How T-cell vaccines and peptide vaccination served to downregulate the autoimmune T-cells and antibodies for the T-cell epitope of hsp65 is not clear. Through an immunostimulatory mechanism, these results indicate that immunoregulatory cells were activated to suppress the autoimmune response to the pancreatic ß-cells, and ultimately prevent IDDM.

Various manipulations of the immune system related to specific antigens have been shown to block the development of IDDM in NOD mice. These include the administration of interleukin 2 (155), treatment with lymphotoxin (156) or OK-432 (157), vitamins such as nicotinamide (50) or 1,25 dihydroxy-vitamin D3 (158), or with immunosuppressive drugs such as CsA (120) or FK-506 (121), insulin administration (159), or treatment with antibodies to T-cells (160,161). Disease prevention in NOD mice can be induced by selective or non-selective action on the immune system as evidenced by these various reports which also indicates that different immune mechanisms may be involved to functionally prevent autoimmune destruction of the pancreatic ß-cells.

PANCREATIC ISLET TRANSPLANTATION IN THE NOD MOUSE

The autoimmune destruction of the pancreatic ß-cells renders the NOD mouse overtly diabetic after 12 weeks of age. As a model of IDDM, islet transplantation in diabetic NOD mice serves as a valuable model to study immune intervention to prevent disease recurrence. The previous section reported many methods by which investigators have successfully prevented the onset of the diabetes and or insulitis in the NOD mouse. Because the NOD mouse inherits the same gender-specific susceptibility genes and the human population at large is heterogeneous in nature it is difficult for investigators to relate studies that prevent IDDM in NOD mice to potential therapeutic intervention in humans (113). On the other hand, reports of methods to prevent the disease recurrence of diabetes after pancreatic islet transplantation in NOD mice are few in comparison and may prove to be more valuable for the population at large that has clinical diabetes and may potentially receive an islet transplant.

In the last ten years there have been many reports of methods to prolong rodent allograft islet transplantation without the need for continuous immunosuppression. Several techniques are described in the literature by which this can be accomplished such as in vitro culture of the donor islets (162), ultraviolet irradiation of the islets to inactivate antigen presenting cells (163), and dissociation of islet preparations into single cell suspensions to obtain purified ß-cells for transplantation (164). In the human islet transplant model, treatment of the allograft response to the transplanted tissue has been largely accomplished with immunosuppressive therapy (25,165). The

potential for graft failure due to rejection or recurrent autoimmune diabetes is still a major barrier to widespread use of islet transplantation (27). It is expected that clinical trails will begin in the near future to access whether immunomodulated islet allografts can be maintained in human recipients without the use of continuous immunosuppression (28). It has yet to be established whether or not the methods outlined above used to alter the expression of rodent allograft islet preparations will be effective in preventing disease recurrence post-islet transplantation in the human transplant model.

The NOD mouse is an ideal model to study the etiology of disease recurrence post islet transplantation. Syngeneic islet transplants under the renal subcapsular space into diabetic NOD recipients leads to a strong immune response which results in disease recurrence and a return to hyperglycaemia (92,166). Previously it has been reported by our laboratory that a single injection of complete Freund's adjuvant (CFA) or bacillus Calmette-Guerin (BCG) given at the time of transplantation of syngeneic islets into diabetic NOD recipients was able to protect the transplanted islets from the autoimmune disease (166,167). The significance of this work is that the autoimmune disease was already present at the time of transplant and the injection of these mycobacterial extracts seemed to have reversed the diabetic process or to have induced a state of tolerance to the transplanted tissue. A recent study by Qin *et al* confirmed the protective effect of CFA as a suppressor of the effector phase of ß-cell destruction and disease recurrence in transplanted islets (133). In cyclophosphamide treated CFA protected animals (spontaneous

disease), the effector cells were activated which lead to the destruction of the ß-cells (133). In NOD mice that exhibit overt diabetes, these effector cells have already been activated by the autoimmune disease to destroy the pancreatic ß-cells. When syngeneic islets are transplanted into diabetic NOD mice with activated effector cells, diabetes recurrence occurs after approximately 15 days of normoglycaemia. CFA, therefore induces a suppressor effect on the effector cells to protect the syngeneic transplanted islets from attack (166). BCG vaccine, when administered at the time of transplantation can also protect islets from autoimmune destruction and, furthermore, when cyclophosphamide is administered to long term protected animals, tolerance to the islet grafts is lost and the effector cells are activated to destroy the transplanted islets resulting in disease recurrence (167,168).

It is not clear what component of either of the two immunoadjuvants, CFA or BCG, is responsible for the protection against the recurrence of disease in islet transplantation. The aim of this thesis was to identify this component by administration of various mycobacterial extracts to NOD mice at the time of syngeneic transplantation. As well, the role of autoantigens in the disease recurrence model was evaluated based on the preliminary evidence reported in the literature of the effectiveness of two antigens, GAD and hsp65, to prevent the spontaneous disease in NOD mice.

II

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Non-obese diabetic mice (K^d, $I\text{-}A^{NOD}$, D^b) were purchased from Taconic Farms (Germantown, NY) and housed in the Health Sciences animal facility (Dentistry-Pharmacy building), University of Alberta. Young NOD males (4-6 weeks old) were used as syngeneic islet donors and females (8-10 weeks old) were used as recipients. The female mice were maintained in variable antigen free (VAF) conditions until onset of diabetes at which point they were transferred to conventional housing. Mice were fed standard laboratory chow and given water *ad libitum*. All animals were cared for following the guidelines of the Canadian Council on Animal Care with the assistance of veterinarians from the Health Sciences Laboratory Animal Services at the University of Alberta.

Older female NOD mice (>8 weeks old) housed in VAF conditions were screened on a weekly basis for the presence of glucose in urine with glucose reagent strips (Tes-Tape, Lilly, Indianapolis, IN). Animals showing glucosuria ($>2^{++}$) had blood glucose levels measured with an ExacTech glucose monitor (MediSense Canada, Mississauga, ON) from blood samples taken from the tail vein. Animals were considered diabetic if non-fasting blood glucose was >16.66 mM. Diabetic females were subsequently transferred to conventional housing for daily monitoring and insulin

administration. Diabetic animals were maintained with a daily subcutaneous injection of one unit of Ultralente beef-pork insulin (Connaught Laboratories, Toronto, ON) for 1-2 weeks prior to islet transplantation.

EXPERIMENTAL PROCEDURES

Islet Isolation

The isolation procedure of the islets of Langerhans is outlined in Figure 1. Islets were harvested from young NOD mice between the ages of four to eight weeks. Mice were anaesthetized with Avertin (2,2,2 Tribromoethanol, Aldrich Chemical Co., Milwaukee, WI) injected intraperitoneally at a dose of 1.5 ml/100g body weight. Once the animals were anaesthetized, a midline incision was made from the xiphoid process to the suprapubic area. With the aid of a Wild Leitz dissecting microscope (magnification 3.2X), the common bile duct was identified and the distal portion of the duct clamped with a mosquito haemostat. Intraductal injection of 2.5 cc of collagenase at 4 °C was achieved by carefully introducing a 27-gauge needle into the common bile duct (Plate I). The collagenase (Type V, Sigma Chemical Co., St. Louis, MO) solution was prepared in advance with cold Hank's Balanced Salt Solution (HBSS) (Gibco, Grand Island, NY) which contained 2.0 mg/ml dextrose (Abbott Laboratories, Montreal, PQ), penicillin (225 U/ml) and streptomycin (225 mg/ml) (Whittaker M.A. Bioproducts, Walkersville, MD). The final concentration of collagenase in the HBSS solution was 2.0 mg/ml. Following intralobular distension of the pancreas, a total

pancreatectomy was performed by rapid dissection from the duodenum. Following excision, the pancreas was placed in chilled HBSS.

Islets were isolated according to the methods outlined by Gotoh et al (88) by stationary digestion. Six to eight NOD pancreases were placed in a 50 ml tissue culture flask (Fisher, Pittsburgh, PA) and an additional 5.0 ml of collagenase solution (2.0 mg/ml) was added. The flask was placed in a 37°C incubator for 32 to 33 minutes. Following the digestion step, cold HBSS was added to the pancreatic extract to inactivate collagenase digestion. The tissue-collagenase solution was vortexed (speed 3) for 10 seconds to further separate the islets from the exocrine tissue. The solution was then centrifuged at 1500 rpm (516 gravities (g)) at room temperature for 10 seconds and the supernatant removed. Three wash steps were conducted using HBSS at 4 °C to remove any remaining collagenase and exocrine debris. Following the final wash, the pancreatic tissue was resuspended in HBSS and poured through a 400 μ filter screen to separate islet tissue from unwanted lymph nodes and fatty tissue (Figure 1). The screen was then sprayed with cold HBSS using a 23-gauge needle to ensure that all islet tissue is collected. A final spin at 1500 rpm (516 g) was performed and pellet resuspended in 10 ml of Ficoll (Type 400-DL, Sigma). Using an automated pipettor and 10 ml serological pipettes, 6.0 ml of 23%, 21.5% and 11% Ficoll solutions were layered carefully in the order of decreasing concentration. See Appendix A for a complete description of Ficoll preparation. The layered Ficoll-tissue solution was then centrifuged for 10 minutes at 2000 rpm (917

g). As exocrine tissue of the pancreas is more dense than the islets themselves, using this discontinuous Ficoll gradient combined with centrifugation allows the islets to be separated from the remaining unwanted pancreatic tissue (79). Following centrifugation, islet tissue was collected from the interface of the 21.5% and the 11% Ficoll layers. Smaller islets most often aggregated in the 23% and 21.5% Ficoll layers interface and were also collected. All islet tissue was removed from Ficoll using a 10-cc syringe and a sterile needle hub from a 14 gauge intravenous catheter/needle unit (Bexton Dickinson, Sandy, UT). Tissue was then washed with room temperature HBSS three times at 1500 rpm (516 g). Following the last wash, all supernatant was aspirated and the pelleted tissue resuspended in 10.0 ml of 25% Ficoll as described earlier. The same procedure for layering the 23%, 21.5% and 11% Ficoll gradients was used. The tissue was centrifuged at 2000 rpm (917 g) for 2 minutes. The isolated islets were removed from the 21.5% and 11% interface and placed in a 50 ml siliconized Pyrex test tube (Fisher). Room temperature Medium 199 (Gibco, Grand Island, NY) which is supplemented with Earl's Salts and 100 mg/L L-glutamine was used to wash Ficoll from the islets. Added to the Media 199 before use was 10% newborn calf serum (Gibco), 25 mM HEPES (N-2-Hydroxyethylpiperazine-N^1-2-Ethanesulfonic acid) (Gibco), penicillin (100 U/ml) (Whittaker) and streptomycin (100 mg/ml) (Whittaker). Islets were washed three to five times with Medium 199 at 1500 rpm (516 g) and placed in a plastic petri dish containing 10 ml of Medium 199. The islets were viewed under a Wild Leitz dissecting

microscope (25X) using a modification of the reflected green light technique of Finke et al (81). Islets, 100 μm - 250 μm in diameter and free of any exocrine debris, were handpicked with a finely drawn Pasteur pipette and counted (Plate II).

Islet Transplantation

The transplantation of the islets of Langerhans is outlined in Figure 2. Animals which were diabetic (>16.6 mM) for one week were used as recipients of islet transplants. No insulin was administered to transplant recipients one day prior to transplantation. On the day of transplantation, animals with a non-fasting blood glucose of >20 mmol/L were used. Anaesthesia was induced with a halothane (2-Bromo-2-chloro-1,1,1-trifluorethane) vaporizer machine and maintained with 1-1.5% halothane in 1 litre of oxygen per minute. A flank incision was made and the right kidney mobilized with a cotton-tipped applicator. A small incision was made in the upper pole of the kidney with a 27-gauge needle. A pouch was created by separating the capsule from the kidney parenchyma with a fine blunt-tipped glass probe toward the lower and anterolateral aspect of the kidney (166). The spin catheter method developed in our laboratory was used to transplant the purified islets in the renal subcapsular space. Four hundred freshly isolated islets of Langerhans (>100 μm in diameter), handpicked free of contaminating exocrine tissue (purity >90%) suspended in medium 199 were brought to the centre of a 10x35 mm petri dish. A micromanipulator syringe (developed at the Surgical-Medical Research Institute, University of Alberta) with

attached 23-gauge needle and 30 cm of polyethylene tubing (PE-50, Fisher) was used to draw up the islets into the tubing which was closed with a Ligaclip (Ethicon, Somerville, NJ). The whole syringe and tubing was spun at 350 rpm for 15 to 20 seconds to pellet the islets at the end of the tubing (Plate III). After the Ligaclip was removed, the tubing was introduced into the small incision formed in the kidney capsule (Plate IV). The islet pellet was slowly advanced into the capsule pouch and the islets prevented from slipping out by sealing the entrance to the pouch with an ophthalmic cautery unit after the tubing had been removed. The islet pellet, now secured under the kidney capsule, was gently spread out using a saline soaked cotton tipped applicator (Plate V). The kidney was gently placed back into the abdomen and the muscle layer closed with 5-0 Dexon II suture (Davis & Geck, Montreal, PQ) using a continuous stitch. The skin layer was closed with wound clips (Clay Adams, Parsippany, NJ). Blood glucose levels were monitored daily post-operation for 3 to 4 days. Transplantation was considered successful if the non-fasting blood glucose returned to normal (<9.4 mM) within 2 to 3 days post-transplant (166,167). Animals that died within the first post-operative week or those that did not become normoglycaemic were excluded from the study and are reported as primary non-function in the results tables.

At the time of transplantation, mice received an injection of the appropriate solution corresponding to the experimental group. The solutions were injected into the hind footpads of the transplant recipient, 50 µl per footpad. This protocol was modelled after that of

Sadelain et al (131).

Metabolic Parameters

All syngeneic islet recipients were housed not more than three mice per cage in conventional housing. The animals were monitored daily and a blood glucose sample taken every second or third day from the tail vein and measured with an ExacTech glucose pen (MediSense). Rejection of the islet graft, following normoglycaemia, was defined as the first of at least two consecutive days of hyperglycaemia (>20 mM).

Nephrectomy and Histology

Rejected graft bearing kidneys of animals that returned to hyperglycaemia (>20 mM) for two consecutive days were removed for histological examination. Graft bearing kidneys of protected animals (>100 days normoglycaemia post-transplant) were removed to confirm conclusively that the transplanted islets were functioning. Due to complications resulting from this procedure, namely liver adherence to the transplanted kidney (particularly to the islet graft site), 50 % of these animals were sacrificed in order to remove the islet graft intact. At this time, if the animal was sacrificed, a portion of pancreas was removed for histological examination to ensure the absence of any insulin staining beta cells. The nephrectomized animals surviving the operation developed immediate onset of hyperglycaemia (>20 mM) and were sacrificed at 2 -3 days post-nephrectomy.

Islet bearing kidneys of non-protected animals (returned to

hyperglycaemia <100 days post-transplant) were fixed in 2% Bouin's solution for 2 hours and then transferred to 70% ethanol. Grafts were processed for paraffin embedding and tissue sections stained with haematoxylin and eosin or Gomori's aldehyde-fuchsin. Islet grafts and pancreas samples from protected animals (>100 days normoglycaemia post-transplantation) were prepared for histological processing either by the method described above or by fixing the samples in O.C.T. compound (Miles Inc., Elkhart, IN), an embedding medium for frozen tissue specimens. The tissue in O.C.T. was frozen in liquid nitrogen and stored in an Ultra-low while awaiting immunohistochemical processing by Dr. Trond Halstenson. Pancreas samples were also processed for paraffin embedding and tissue stained with Gomori's aldehyde-fuchsin. Histological samples were reviewed in this case by a clinical pathologist, Dr. T. Shnitka, without knowledge of the specimen source and graded 1+ to 4+ based on the degree of lymphocytic infiltration and insulitis with 0 equalling no infiltrate and 4+ heavy infiltrate indicating complete destruction of the ß-cells. The degree of peri-insulitis in the histological samples were also graded by Dr. Shnitka on a 0 to 4+ grading system with 0 equalling no peri-insulitis and 4+ massive accumulation of lymphocytic infiltration surrounding the transplanted islets.

STATISTICAL ANALYSIS

Differences in islet graft survival were evaluated by the Wilcoxan rank sum test using the SPSS-PC statistical software, a

nonparametric procedure utilized with two samples to test the null hypothesis that the distributions of two variables are the same. The absolute values of the differences between two variables are calculated for each use and ranked from smallest to largest. The test statistic is based on the sums of ranks for negative and positive differences (169). This test was employed to test differences in graft survival lengths in the experimental groups of animals. The null hypothesis is that the two samples come from populations having the same distribution was rejected if two samples were significantly different at <0.05.

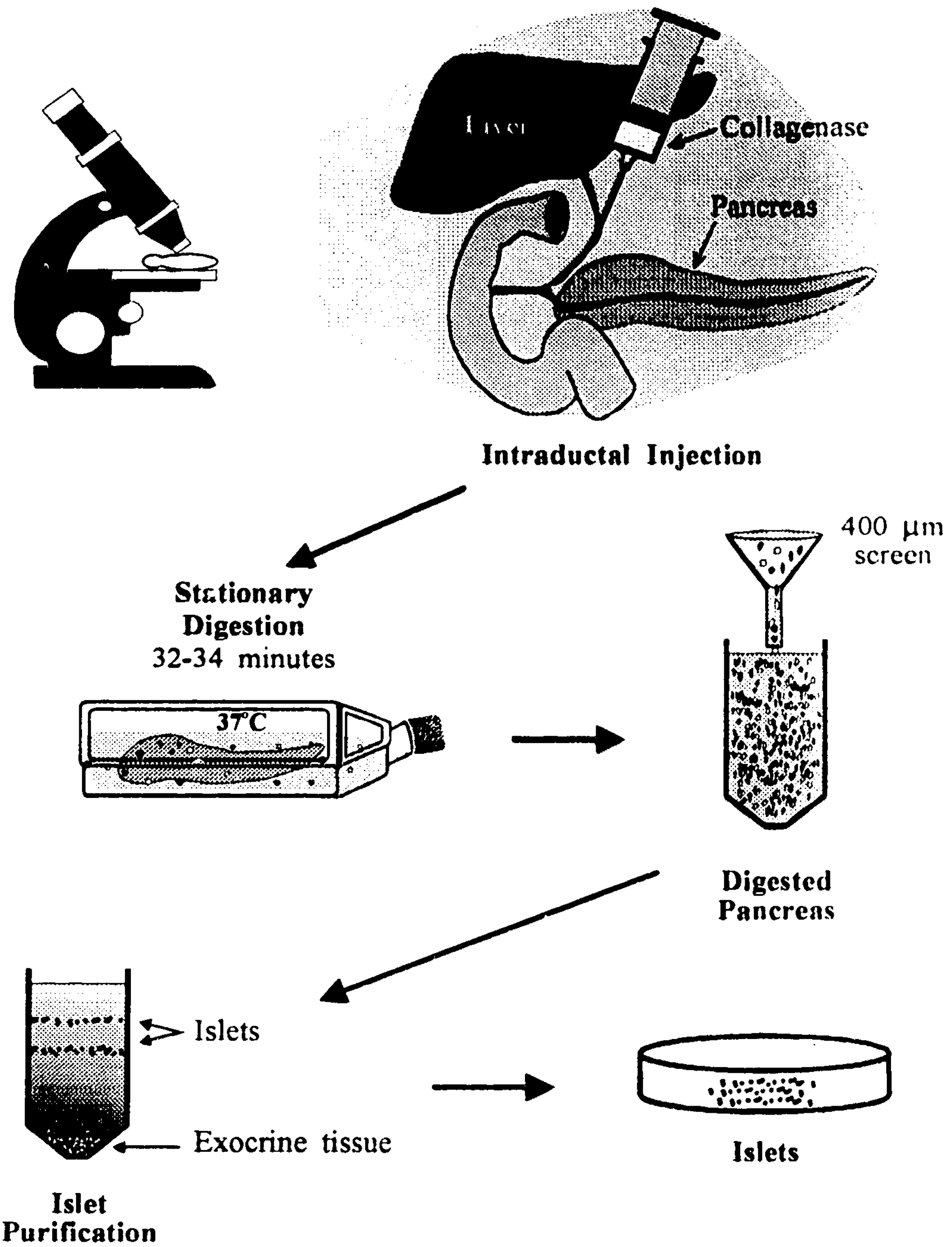


Figure 1. ***Isolation of mouse pancreatic islets by the stationary digestion method.*** This figure shows the intraductal administration of collagenase and purification steps.

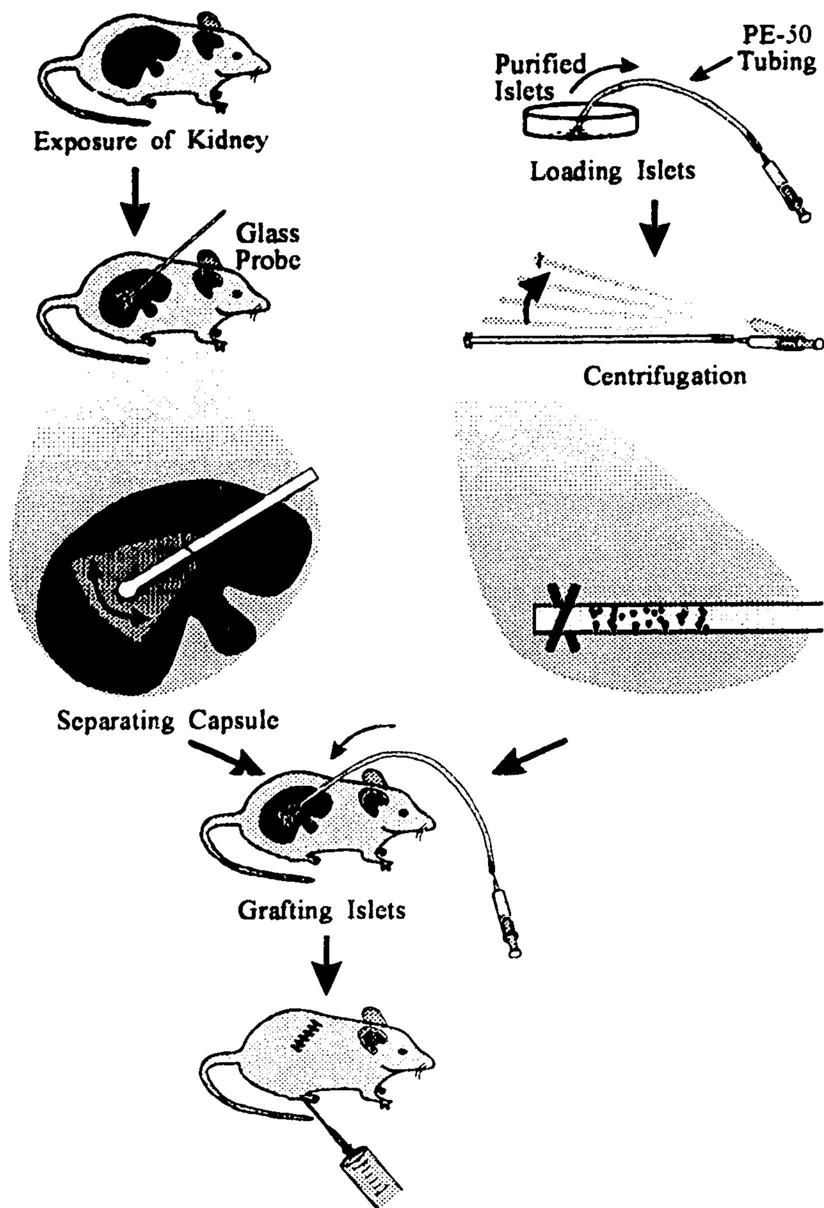


Figure 2. ***Islet transplantation in the mouse by the renal capsule method.*** This figure shows (on the left) formation of a pocket under the renal caspsule. The loading of the islets by the micromanipulator and the spinning catheter method of islet concentration is shown on the right. When these steps are complete, the islets are slowly injected under the renal capsule and the kidney replaced into the abdomen, after which the animal is injected in each of the hind footpads with one of the experimental treatments.

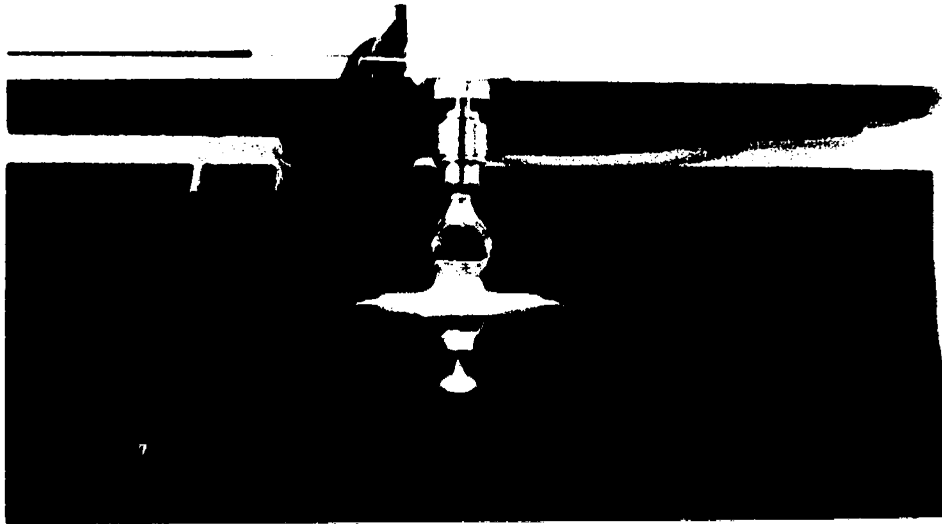
I

II

Plate I: Pancreas distension by the intoduction of collagenase solution through the common bile duct

Plate II: Freshly isolated islets of Langerhans from a prediabetic NOD mouse (original magnification x 175)

III



IV

V

Plate III: Spinning catheter method of collecting islets of Langerhans for transplantation.

Plate IV: Introduction of islets under the renal capsule in a diabetic NOD mouse.

Plate V: Completion of islet transplantation procedure: islet graft before replacing kidney into abdomen.

III
RESULTS

EXPERIMENTAL GROUPS

Group 1: Complete Freund's Adjuvant (CFA)

The objective of this set of experiments was to confirm the effectiveness of CFA (DIFCO, Detroit, MI) in protecting syngeneic islets from disease recurrence post-islet transplantation. An emulsion of CFA (containing *Mycobacterium tuberculosis*) or incomplete Freund's adjuvant (IFA) (DIFCO) in physiological saline were prepared new for each injection. Fifty μl of CFA or IFA was diluted with 50 μl of saline and vigorously mixed using a vortex. A total volume of 100 μl (50 μl each footpad) was injected into the hind footpads at the time of islet transplantation. Control mice were injected with 100 μl of saline (50 μl each footpad) and another group of control animals did not receive footpad injections.

Table 1 (page 57) shows graft survival for these four groups of animals. CFA gave extended graft survival which was similar to the published results of Wang *et al* (166). The majority of the animals in this group had graft function past 100 days however 6 animals did lose their graft function before 50 days due to disease recurrence. The overall mean graft survival for this group of animals was 90.5 days which was significantly longer than the IFA, saline and non-treated controls. The IFA group had a range of graft survival from 15

days to 32 days with one animal going out to 76 days before returning to hyperglycaemia. The mean graft survival for the IFA group was 27.8 which was not significantly different than the saline and non-treated controls which had mean graft survivals of 19.2 days and 15.7 days respectively. Figure 3 (page 58) shows this same data as graft survival versus days post-transplant. This figure clearly shows that the non-treated controls all rejected their grafts by 23 days whereas the saline-injected animals had 10% (n = 1) of the animals going out to 38 days. The majority of the IFA group rejected their grafts by day 32 with one animal going out to 76 days. The CFA group had 70% of the animals that went long term past 100 days which was significantly longer than the other three groups of animals. Individual blood glucose profiles of animals in each of the groups are located in Appendix B (pages 103-106).

To confirm that the transplanted islets were functioning, eleven CFA protected mice whose graft survival extended past 80 days had the islet-bearing kidney removed as shown in Table 1. The removed islet-bearing kidney was given to Dr. Halstenson who is presently identifying the cell phenotype of the peri-insular infiltrate surrounding the transplanted islets. Animals surviving this difficult operation (n = 6) immediately became hyperglycaemic (>20 mM) proving that the transplanted islets and not residual ß-cells in the pancreas were responsible for maintaining euglycaemia. Preliminary results (personal communication with Dr. Halstenson) from the histological examination of the islet-bearing kidney grafts from CFA protected animals showed well-granulated islets with no intra-islet

insulitis and variable peri-insular lymphocytic infiltration (1+ to 3+). Histological examination of the pancreases from CFA protected animals that did not survive the nephrectomy (n = 5) confirmed the absence of endogenous insulin-containing ß-cells (Plates VI,VII, page 59). These results confirmed therefore that the maintenance of normoglycaemia was due to the engrafted islets rather than residual ß-cells in the pancreas which had been destroyed by the autoimmune process. Histological examination of engrafted kidneys from non-protected animals of the IFA, saline and untreated treatment groups showed atrophic islets with minimal or no aldehyde-fuchsin staining (Plates VIII,XI, pages 60,61). Histological examination of all grafts undergoing disease recurrence revealed extensive-to-complete islet destruction which confirms the disease process is still activated at the time of islet transplantation.

Group 2: Mycobacterium bovis, Escherichia coli and Salmonella typhimurium Bacteria and Lysates

At the time that the following experiments were initiated, it was believed that the autoantigen glutamic acid decarboxylase (GAD) was present in *Mycobacterium bovis* which makes up bacillus Calmette-Guerin (BCG) vaccine. Speculation of the role of GAD in the protective effect of BCG in the disease recurrence model of islet transplantation prompted this set of experiments. Three bacterial strains (*M. bovis, E. coli, S. typhimurium*) were evaluated for their protective effect in the islet transplantation model. *Mycobacterium bovis* (BCG) as stated previously was believed to express GAD,

Escherichia coli bacteria is an over-expresser of the antigen GAD (170), and *S. typhimurium* bacteria does not express GAD and therefore served as a control for *M. bovis* and *E. coli*. Two different bacterial preparations (as whole bacteria or as a lysate) were evaluated. BCG Pasteur strain, *E. coli* strain W and *Salmonella typhimurium* LT2 bacterial cultures were grown in Dr. John Elliott's laboratory (Department of Medical Microbiology and Infectious Diseases, University of Alberta). Whole bacteria was prepared by autoclaving and lyophilizing to produce a powder which was diluted with IFA and water to a concentration of 2.0 mg/ml. Bacterial lysates were produced by running rinsed bacteria through a French Press to lyse the cells and the lysates were stored at -70 °C. Aliquots of the lysates were freeze-dried to determine dry equivalent weight per volume of lysate. Before use, the freeze-dried lysates were resuspended at an equivalent concentration of 2.0 mg/ml in IFA and water. At the time of syngeneic islet transplant, 50 µl of the appropriate bacteria or lysate was injected into the left hind footpad of the transplant recipients. The right hind footpad was injected with 50 µl of saline. A comparison was made between the degree of swelling in the left footpad as compared to the right versus the length of islet graft protection. This comparison produced no conclusive results and will not be reported in this thesis.

The mean graft survival for these six groups of animals is shown in Table 2 (page 62) and the individual blood glucose profiles are located in Appendix C (pages 107-112). Within these six groups of animals, there was no significant difference in the time for disease

recurrence post-transplantation as determined by the Wilcoxan rank sum test. Animals injected with *E. coli* bacteria, *E. coli* lysate, *S. typhimurium* bacteria and *S. typhimurium* lysate all returned to hyperglycemia by day 55. Two animals, one from each of the BCG bacteria and BCG lysate treatment groups, did have extended graft survival to 125 and 122 days respectively at which time hyperglycaemia ensued indicating that the autoimmune disease was still present and was delayed from destroying the transplanted islets. With the exception of these two animals, the rest of the transplanted animals in the BCG bacteria and lysate groups all lost their grafts by 63 days. These results indicated that whether GAD is present (*E. coli* and BCG) or not (*S. typhimurium*), graft survival is not extended. In comparing these 6 groups of animals to the saline and non-treated controls there was a significant prolongation of graft survival however this survival was not different than the IFA group. Because there was no statistical significance in graft survival between the six groups receiving bacteria or lysate and the IFA group, no definite conclusion can be made because the bacterial and lysate treatments were all mixed in IFA as the injection vehicle. It is interesting to note that at the end of this study it was confirmed by Dr. Elliott's lab that there was no GAD present in BCG vaccine thus ruling out the possibility that GAD was the protein responsible for the protection of the transplanted islets by live BCG vaccine.

Group 3: Mycobacterium bovis and Escherichia coli

In an effort to isolate the component(s) of *Mycobacterium* responsible for the protective effect on syngeneic transplanted islets, a further set of experiments was carried out. It was speculated that the same concentration of heat-killed *M. bovis*, the strain of bacterium which constitutes BCG vaccine, would have the same effect as CFA if immersed in IFA, the oil component of CFA. Two concentrations of *M. bovis* were selected, 50 μg, the dose used to immunize mice with CFA (*M. tuberculosis*) and 2.7 mg, the dose required to immunize mice with the live BCG vaccine (167).

BCG, an attenuated strain of *Mycobacterium bovis*, was purchased from Connaught Laboratories (Toronto, ON). This material comes as a live freeze-dried powder in either a 27 mg/vial (ImmunoCyst) or a 1.5 mg/vial (BCG vaccine) form. Both products were used depending on the desired final concentration. *Escherichia coli* (*E. coli*) strain W bacterial culture was grown in Dr. John Elliott's laboratory. The BCG and *E. coli* preparations were killed by repeated steps of autoclaving and freeze-drying and were then diluted with saline and IFA. At the time of syngeneic islet transplantation, 100 μl of dead BCG or *E. coli* (50 μg or 2.7 mg) was injected into the hind footpads of the transplant recipients, 50 μl per footpad. Individual blood glucose profiles of animals in each of the groups are located in Appendix D (pages 113-116).

Table 3 (page 63) shows graft survival for heat-killed *M. bovis* and *E. coli* at 50 μg and 2.7 mg doses. Mice immunized with *M. bovis* at 2.7 mg had a range of graft survival from 30 to 62 days with

an overall mean of 45.6 days which was lower than the published mean graft survival of the live BCG protected animals (>100 days) (167). The range of graft survival for the group of animals receiving 2.7 mg of dead *M. bovis* was however significantly longer than the IFA, saline and non-treated animals (see Table 1). The same dose of *E. coli* (2.7 mg) administered to mice at the time of islet transplantation did not extend graft survival time. Histological samples from animals treated with 2.7 mg of *M. bovis* or *E. coli* showed islet infiltration by mononuclear cells and ß-cell degranulation (Plates X,XI, pages 64,65). In fact, the four treatments used to immunize transplanted mice in this experiment were not significantly different when compared with each other, however there was a significant prolongation of graft survival as compared to the saline and non-treated animals (Taele 3). Two mice from the group of animals treated with *M. bovis* at 50 µg did have extended graft survival past 100 days. At 112 and 152 days respectively the graft was removed for histological examination. Histological examination of these engrafted kidneys revealed no insulitis and 2+ peri-insular infiltrate surrounding the transplanted islets (Plates XII, page 66). Four of seven animals from the same group (*M. bovis* 50 µg) did not reach 50 days graft survival therefore despite the two animals in this group that did have extended graft survival, these results do not confirm any signifi‹ant long term protective effect from disease recurrence in animals treated with dead *M. bovis* at 50 µg. No treatment in this experiment resulted in long term graft survival when compared to the protective effect of 50 µg of *M. tuberculosis* (CFA)

demonstrated in Group 1 or the protective effect of 2.7 mg of live BCG vaccine reported by Lakey *et al* (167).

Group 4: Mouse GAD67 and Human GAD65

Recent studies have indicated that T-cell tolerance to the ß-cell autoantigen GAD is achieved by the intravenous or intrathymic administration of recombinant GAD65 into young NOD mice (147,148). These studies have shown that the incidence of insulitis and diabetes is suppressed in animals receiving GAD65. Because GAD has been speculated as a putative autoantigen in ß-cell destruction, it was decided to examine the effectiveness of this protein in the prevention of disease recurrence of diabetes post-islet transplantation. One of two isoforms of GAD, mouse GAD67 or human GAD65, was administered at the time of islet transplantation. Using these proteins it was hypothesized that the T-cell response to GAD and other antigens would be suppressed thus resulting in protection of the transplanted islets from diabetes recurrence.

Syngeneic islet transplantation in this group of animals was followed immediately by a foot pad injection of either purified mouse GAD67, PFSY-A1 (a similarly purified control protein derived from *Plasmodium falciparum*), or a human GAD65 peptide. These reagents were provided by Dr. John Elliott. Prior to islet transplantation, the reagents were dissolved in saline and emulsified in IFA (a final dose of 50 μg of protein was administered to each transplant recipient). The final volume of injectable was 100 μl, 50 μl in each hind footpad.

The mean graft survivals for the groups of animals in this experiment are shown in Table 4 (page 67) and the individual blood glucose profiles of animals in each of the groups are located in Appendix E (pages 117-119). Mice treated with mouse GAD67 had a range of graft survival from 20 days with two animals going out past 100 days. The overall mean for this group of animals was 44.6 days which was not significantly longer than the human GAD65 and control protein groups. The range of graft survival in the mouse GAD67 group was significantly longer when compared to graft survival in the saline and non-treated controls from Table 1 but was not significantly different than the range of graft survival in the IFA group also from Table 1. The human GAD65 and control protein groups had a mean graft survival of 50.3 and 49.6 days respectively. Again, two animals in each of these groups went past 100 days which skewed the mean graft survival. Human GAD65 and control protein groups did not have a range of graft survival significantly different than the IFA or saline groups from Table 1. Figure 4 (page 68) shows graft survival versus days post-transplant. For comparison, the IFA, saline and non-treated controls were included in this figure. As can be seen from Figure 4, there was a slight prolongation of graft survival with approximately 30% of mice in each of the GAD65 and GAD67 groups showing long term acceptance of their islet grafts past 100 days. This result was not significantly different than the control protein as can be seen in Figure 4.

Nephrectomies were performed on all mice with graft survival past 100 days to confirm function of the transplanted islets.

Histological examination of the engrafted kidneys from protected animals (>100 days normoglycaemia post-transplantation) that received GAD65, GAD67 and PFSY-A1 showed moderate peri-insulitis from 1+ to 3+ and little or no islet infiltration by mononuclear cells (personal communication with Dr. Halstenson). Histological examination of the animals in the GAD injected groups that did not show long term survival revealed severely atrophic islets heavily infiltrated with mononuclear cells, an accurate measure of ß-cell destruction (Plate XIII, page 69).

Group 5: Heat Shock Proteins

Vaccination against murine IDDM has been successful with hsp65 peptide (153,154). Elias et al utilized the 65kDa heat shock protein of *M. tuberculosis* (hsp65-Mt) to vaccinate young NOD mice with a single injection of 50 μg of peptide emulsified in IFA. The decreased incidence of diabetes reported in this study indicated that the spontaneous T-cell reactivity to hsp65 was suppressed and the spontaneous autoimmune process of ß-cell destruction arrested. Because of these published results and our intention to develop strategies to prevent the disease recurrence post-islet transplantation, heat shock proteins were examined for their effectiveness in protecting syngeneic islets transplanted in a diabetic NOD mouse.

M. bovis (hsp65-Mb), *M. tuberculosis* (hsp70-Mt) and *M. leprae* (hsp65-Ml) derived heat shock proteins were kindly received from Dr. J.D.A. van Embden at the National Institute of Public Health and Environmental Hygiene, The Netherlands (Financial support from the

UNDP/World Bank/WHO Special programme for Research and Training in Tropical Diseases). Each protein was dissolved in an equivalent volume of saline and at the time of syngeneic islet transplant, 50 μg of protein was combined 1:1 with IFA and 100 μl injected (50 μl each footpad) into the hind footpads of the transplant recipients.

Table 5 (page 70) shows that animals receiving a single footpad injection of the hsp65-Ml (*M. leprae*) had a range of graft survival from 22 days with the majority of these animals going past 90 days. The range of graft survival for the *M. leprae* treated animals was significantly longer than the range of graft survival in the *M. bovis* and *M. tuberculosis* groups of animals. The range of graft survival in the *M. bovis* hsp group was 16 days to 64 days with one of seven animals having extended graft survival past 100 days. The range of graft survival in the *M. tuberculosis* hsp group was 13 days to 61 days with a mean graft survival of 27.3 days, much lower than the *M. leprae* group. In Figure 5 (page 71) which shows graft survival versus days post-transplant, it is clear to see that only the hsp derived from *M. leprae* gave extended graft survival as compared to the IFA, saline and non-treated controls. Sixty percent of the animals in the *M. leprae* group had graft survival past 100 days compared to only 15% of the animals in the *M. bovis* group and no animals extending past 61 days in the *M. tuberculosis* group. Individual blood glucose profiles of animals in each of the groups is located in Appendix F (pages 120-122).

Four of seven animals in the *M. leprae* group whose islet grafts functioned long term past 100 days underwent nephrectomies to confirm islet graft function. Two animals from the *M. leprae* group survived the operation and returned to hyperglycaemia post-nephrectomy. Histological examination of the pancreas and engrafted kidneys from *M. leprae* derived hsp protected animals that did not survive the nephrectomy operation (n = 2) confirmed the absence of endogenous insulin-containing ß-cells in their pancreas and their presence under the kidney capsule (data not shown). These results confirm that the maintenance of normoglycaemia was due to the engrafted islets rather than residual ß-cells in the pancreas which had been destroyed. Histological examination of the islet-bearing kidneys from the *M. leprae* derived hsp protected animals (n = 4) showed no mononuclear cell infiltration (0 insulitis) and very limited peri-insulitis (1+ to 2+) and positive staining for insulin determined by the grade of aldehyde-fuchsin staining (Plate XIV, page 72). These results confirm that the islets protected from the disease recurrence by *M. leprae* derived hsp were intact and functioning.

Table 1. Syngeneic islet graft survival in NOD mice treated with adjuvant therapy.

Group	N (PNF)	Individual Graft Survival Time (days)	Mean ± SEM	Median	*P* value[a] vs CFA
CFA	17 (0)	16, 27x2, 29, 33, 48, 84*, 92*, 92*, 114*, 127*, 129*, 131*, 133*, 139*, 156*, 161*	90.5± 12.0	88	--
IFA	9 (1)	15x2, 16x2, 25, 26, 29, 32, 76	27.8 ± 6.4	25	0.001
Saline	11 (0)	12, 14,15x4, 19, 20x2, 28, 38	19.2 ± 2.3	15	<0.001
Untreated	13 (6)	8x2, 12, 13, 15x3, 16x2, 18, 22, 23x2	15.7 ± 1.4	15	<0.001

* Denotes nehrectomy

[a] Wilcoxon rank sum test

(PNF) animals with primary non-function of their islet graft and not returning to normoglycemia (>9.4 mmol/L) within 2 to 3 days post-transplant and therefore not included in the study.

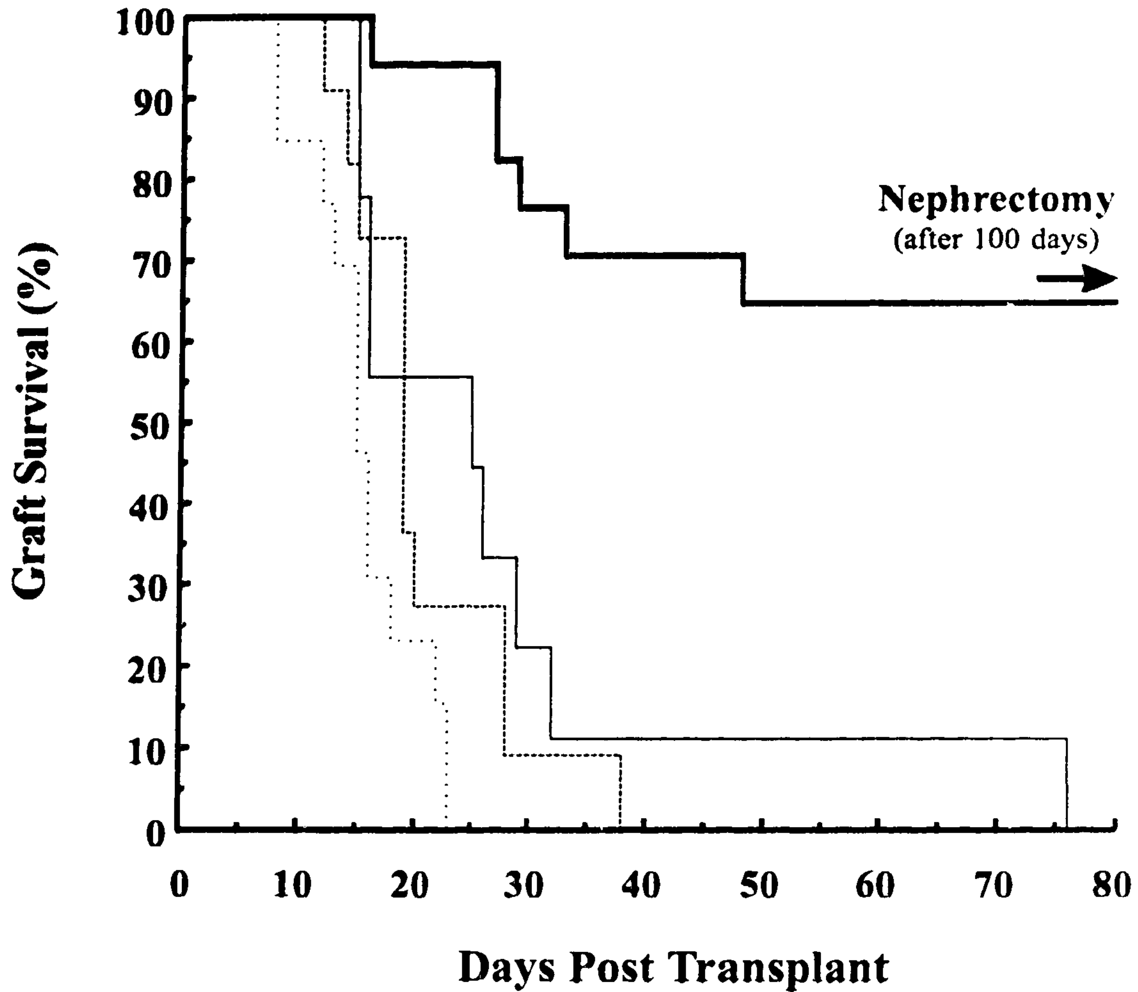
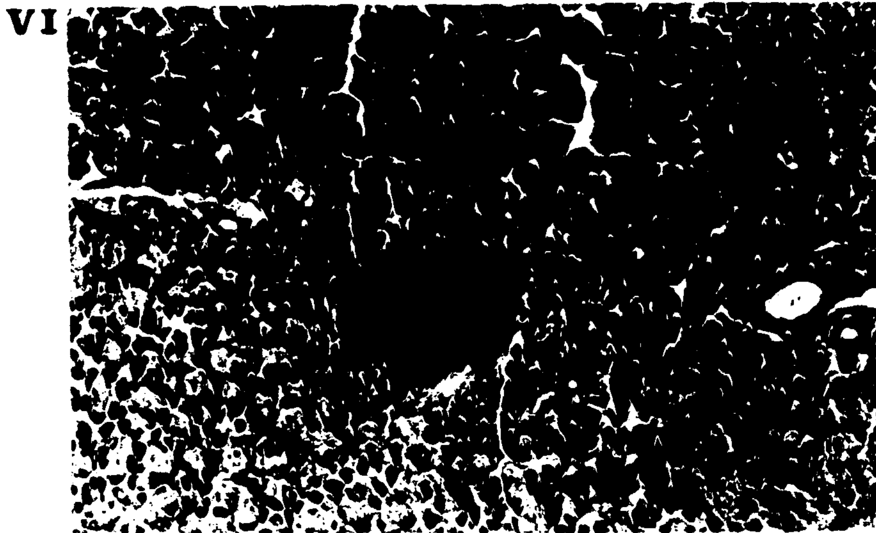


Figure 3. ***Time course of the prevention of recurrence of diabetes in NOD mice receiving islet transplants and adjuvant therapy.*** Female mice receiving injections of CFA in the hind footpads at the time of transplantation (n=17) (——); mice receiving IFA (n=9) (—) or saline (n=11)(------) in the hind footpads at the time of transplantation; mice receiving islets without footpad injections (n=13)(· · ·).

VI



VII

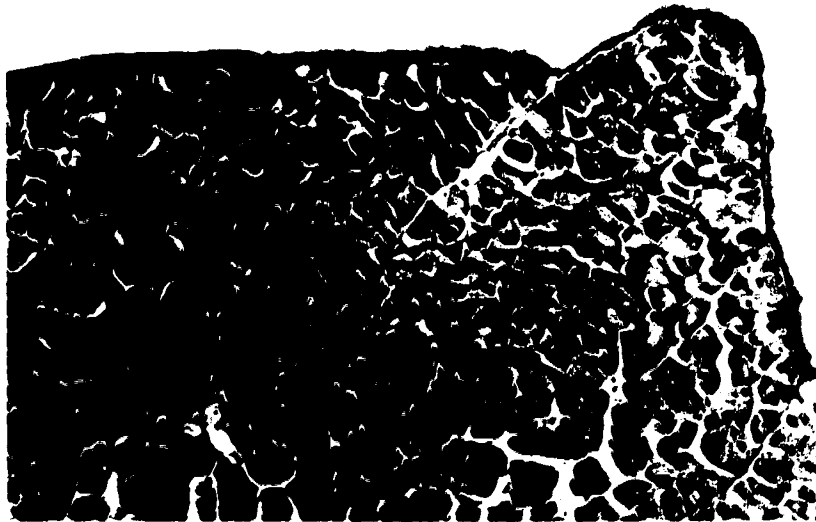
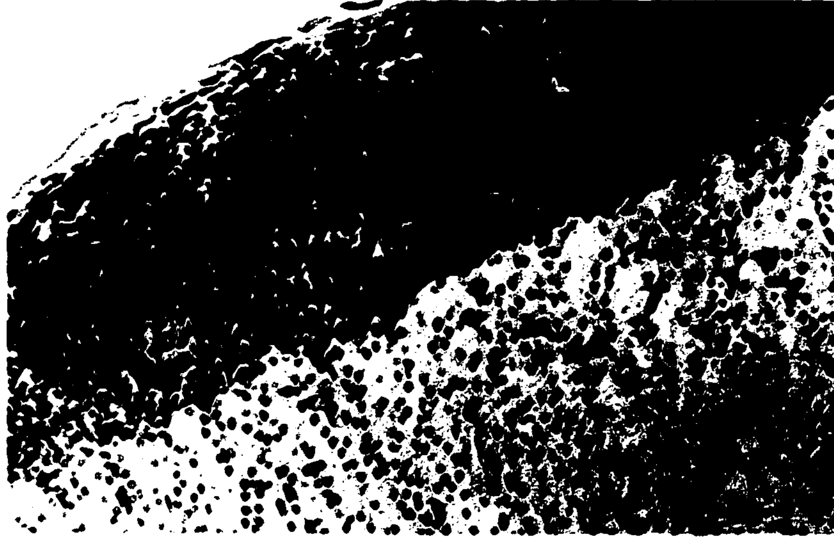


Plate VI: Pancreatic islets (aldehyde-fuchsin stain) of a non-diabetic NOD age 5 to 7 weeks. The islets show well-granulated beta cells (dark stain) with no evidence of insulitis or peri-insular lymphocytic infiltration of mononuclear cells (original magnification x 200).

Plate VII: Pancreatic section (aldehyde-fuchsin stain) from an animal immunized with complete Freund's adjuvant (CFA) that received a nephrectomy at 136 days post-transplant and did not survive the operation. Note that no islets of Langerhans are present (original magnification x 200)

A



B

Plate VIII: Photomicrographs of syngeneic islet grafts transplanted under the renal capsule of a diabetic NOD mouse that received incomplete Freund's adjuvant at the time of transplantation and developed hyperglycemia 16 days post-transplant. The graft was removed on day 2 after the onset of hyperglycemia. (**A**) hematoxylin-eosin stained section showing heavy peri-insular and intraislet infiltration of mononuclear cells. (**B**) aldehyde-fuchsin stained section showing no staining indicating beta-cell destruction (original magnification x 200).

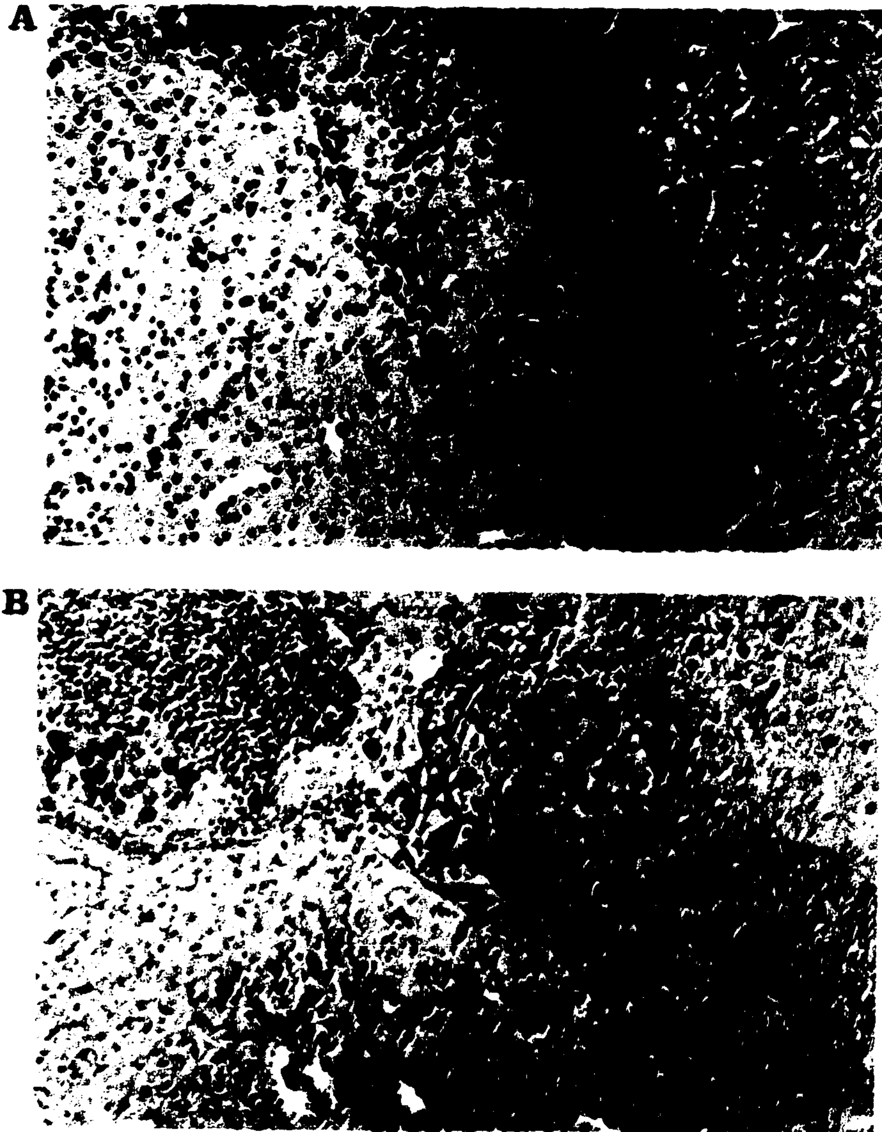


Plate IX: Photomicrographs of syngeneic islet grafts transplanted under the renal capsule of a diabetic NOD mouse that received incomplete Freund's adjuvant at the time of transplantation and developed hyperglycemia 32 days post-transplant The graft was removed on day 1 after the onset of hyperglycemia (**A**) hematoxylin-eosin stained section of islets becoming eroded by infiltrating mononuclear cells. (**B**) aldehyde-fuchsin stained section showing markedly reduced intensity of staining indicating beta-cell degranulation (original magnification x 200)

Table 2. Syngeneic islet graft survival in NOD mice treated with bacteria and lysates.

Groups	N (PNF)	Individual Graft Survival Time (days)	Mean ± SEM	Median	*P* value[a] vs I & II	III
BCG Bacteria	5 (4)	22, 23, 42, 44, 125	51.2 ± 19.0	42	<0.05	N.S.
BCG Lysate	7 (2)	17, 27, 28x2, 30, 63, 122	45 ± 13.9	28	<0.05	N.S.
Escherichia coli Bacteria	5 (1)	19, 27, 30, 35, 54	33 ± 5.6	30	<0.05	N.S.
Escherichia coli Lysate	4 (3)	19, 23, 29, 36	26.8 ± 3.7	26	<0.05 (II only)	N.S.
S. typhimurium Bacteria	5 (1)	20, 22, 28, 31, 48	29.8 ± 5.0	28	<0.05	N.S.
S. typhimurium Lysate	5 (2)	28, 33, 42, 43, 46	38.4 ± 3.9	42	<0.05	N.S.

[a] Wilcoxon rank sum test

I vs Saline (Table 1)

II vs Untreated (Table 1)

III vs IFA (Table 1)

(PNF) animals with primary non-function of their islet graft and not returning to normoglycemia (>9.4 mmol/L) within 2 to 3 days post-transplant and therefore not included in the study.

Table 3. Syngeneic islet graft survival in NOD mice treated with *Mycobacterium bovis* or *Escherichia coli*.

Groups (dose)	N (PNF)	Individual Graft Survival Time (days)	Mean ± SEM	Median	*P* value[a] vs	
					I & II	III
M. bovis (2.7 mg)	7 (0)	30, 34, 42, 48x2, 55, 62	45.6 ± 4.3	48	<0.05	0.01
M. bovis (50 μg)	7 (0)	15x2, 17, 42, 74, 112*, 152*	61 ± 20.5	42	<0.05	N.S.
E. coli (2.7 mg)	6 (1)	20, 23, 36, 41, 51, 55	37.7 ± 5.8	38.5	<0.05	N.S.
E. coli (50 μg)	6 (2)	20x2, 23, 24, 42, 108	39.5 ± 14.1	23.5	<0.05	N.S.

* Denotes nephrectomy

[a] Wilcoxon rank sum test

I vs Saline (Table 1)

II vs Untreated (Table 1)

III vs IFA (Table 1)

(PNF) animals with primary non-function of their islet graft and not returning to normoglycemia (>9.4 mmol/L) within 2 to 3 days post-transplant and therefore not included in the study.

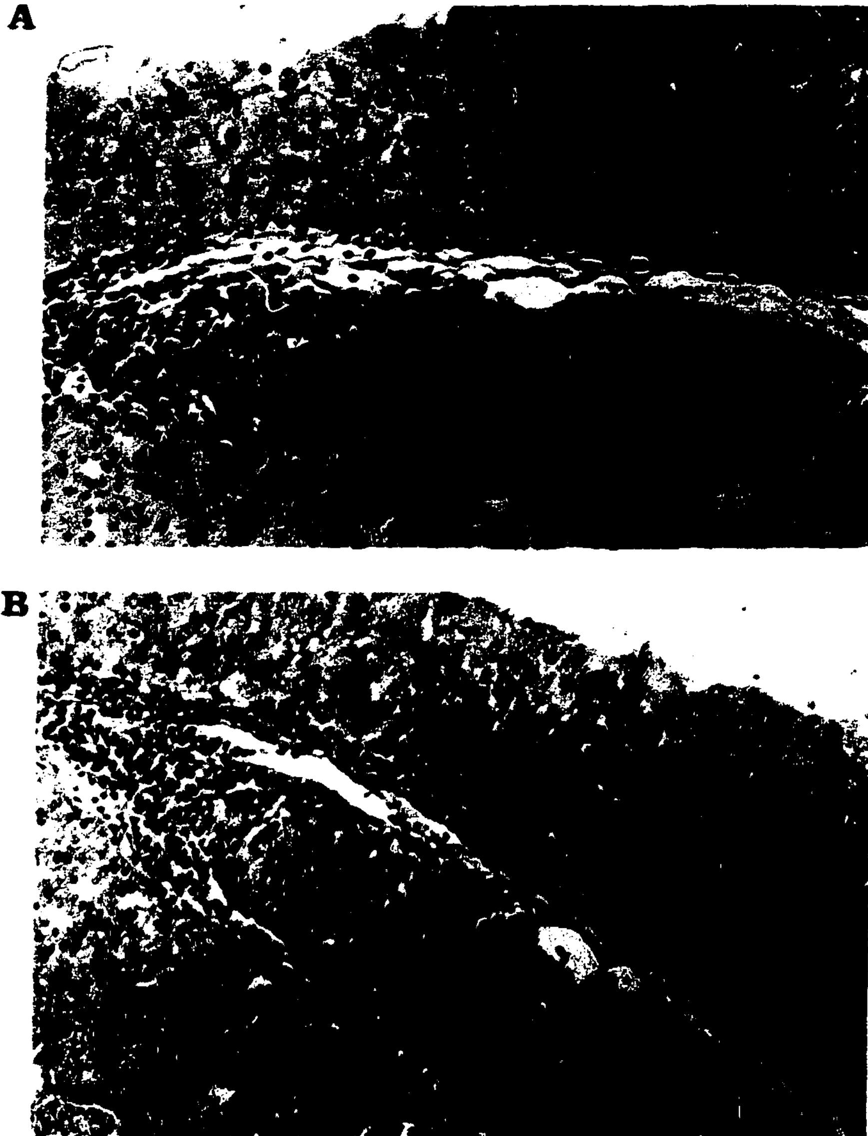
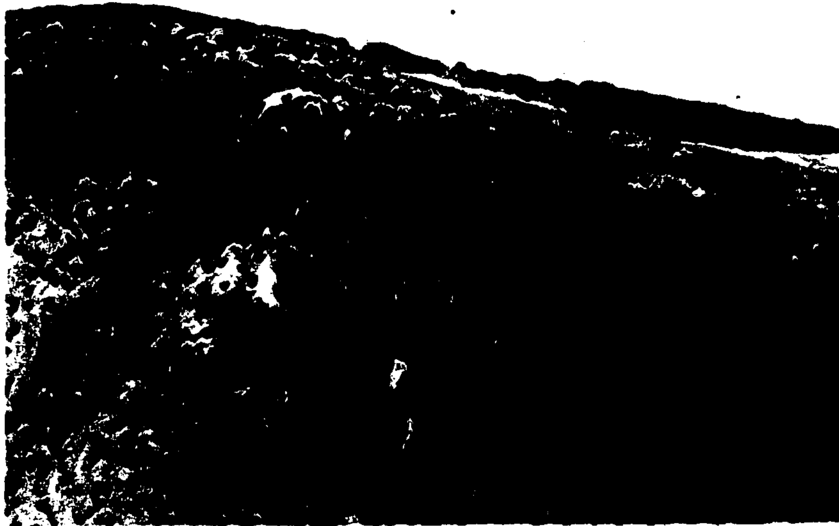


Plate X: Photomicrographs of syngeneic islet grafts transplanted under the renal capsule of a diabetic NOD mouse that received 2.7 mg of killed *Mycobacterium bovis* at the time of transplantation and developed hyperglycemia 30 days post-transplant. The graft was removed on day 2 after the onset of hyperglycemia. (**A**) hematoxylin-eosin stained section showing moderate sized islets infiltrated by mononuclear cells. (**B**) aldehyde-fuchsin stained section showing beta-cell degranulation and no staining (original magnification x 200)

A



B

Plate XI: Photomicrographs of syngeneic islet grafts transplanted under the renal capsule of a diabetic NOD mouse that received 2.7 mg of killed *Escherichia coli* at the time of transplantation and developed hyperglycemia 51 days post-transplant. The graft was removed on day 2 after the onset of hyperglycemia. (**A**) hematoxylin-eosin stained section showing islets becoming eroded by the intra-islet infiltration of mononuclear cells. (**B**) aldehyde-fuchsin stained section showing beta-cell degranulation and no staining (original magnification x 200).

Plate XII: Photomicrographs of syngeneic islet grafts transplanted under the renal capsule of a diabetic NOD mouse that received 50 μg of killed *Mycobacterium bovis* at the time of transplantation and remained normoglycaemic until receiving a nephrectomy at 122 days post-transplant. **(A)** hematoxylin-eosin stained section showing a large islet surrounded but not infiltrated by mononuclear cells. **(B)** aldehyde-fuchsin stained section showing well-granulated beta-cells as indicated by the dark stained areas (original magnification x 200).

Table 4. Syngeneic islet graft survival in NOD mice treated with glutamic acid decarboxylase.

Groups	N (PNF)	Individual Graft Survival Time (days)	Mean ±SEM	Median	*P* value[a] vs I	II
Mouse GAD67	10 (0)	20, 21, 23x2, 24, 27, 28, 33, 114*, 133*	44.6 ± 13.3	25.5	0.014	<0.001
Human GAD65	7 (0)	13, 16, 20, 23, 43, 112*, 125*	50.3 ± 18.0	23	N.S.	0.022
PFSY-A1	7 (1)	14, 15, 17, 21, 54, 112*, 120*	50.4 ± 17.7	21	N.S.	0.022

* Denotes nephrectomy

a Wilcoxon rank sum test

I vs Saline (Table 1)

II vs Untreated (Table 1)

(PNF) animals with primary non-function of their islet graft and not returning to normoglycemia (>9.4 mmol/L) within 2 to 3 days post-transplant and therefore not included in the study.

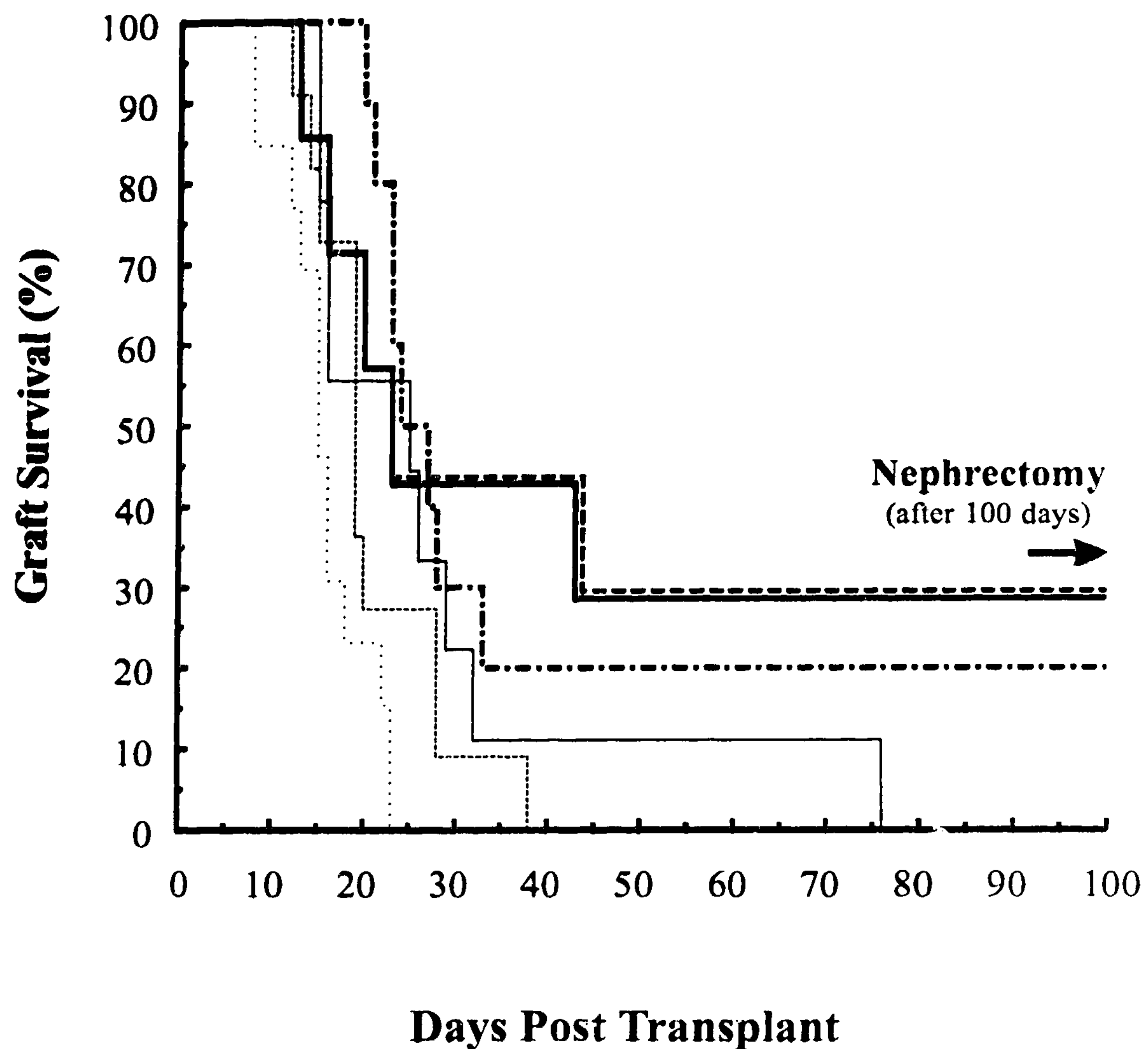


Figure 4. ***Time course of the prevention of recurrence of diabetes in NOD mice receiving islet transplants and GAD.*** Female mice receiving injections of GAD67 in the hind footpads at the time of transplantation (n=10) (-•-); mice receiving injections of GAD65 in the hind footpads at the time of transplantation (n=7)(---); mice receiving PFSY-A1 (n=7) (—), IFA (—) (n=9) or saline (n=11)(------) in the hind footpads at the time of transplantation; mice receiving islets without footpad injections (n=13)(···).

A

B

Plate XIII: Photomicrographs of syngeneic islet grafts transplanted under the renal capsule of a diabetic NOD mouse that received 50 μg of human GAD65 peptide at the time of transplantation and developed hyperglycemia 23 days post-transplant. The graft was removed on day 2 after the onset of hyperglycemia. **(A)** hematoxylin-eosin stained section showing severely atrophic islets heavily infiltrated by mononuclear cells. **(B)** aldehyde-fuchsin stained section showing beta-cell degranulation and no staining (original magnification x 200).

Table 5. Syngeneic islet graft survival in NOD mice treated with heat shock proteins.

Groups	N (PNF)	Individual Graft Survival Time (days)	Mean ± SEM	Median	*P* value[a] vs *M. leprae*
hsp65 kD *M. leprae*	7 (1)	22, 48, 87, 118*, 131*, 138*, 146*	98.6 ± 18.1	118	--
hsp65 kD *M. bovis*	7 (0)	16, 19, 20, 21, 38, 64, 131*	44.1 ± 15.8	21	0.030
hsp70 kD *M. tuberculosis*	11 (1)	13, 18, 19x2, 20, 21, 27x2, 29, 46, 61	27.3 ± 4.3	21	0.003
IFA[b]	9 (1)	15x2, 16x2, 25, 26, 29, 32, 76	27.8 ± 6.4	25	0.007
Saline[b]	11 (0)	12, 14,15x4, 19, 20x2, 28, 38	19.2 ± 2.3	15	0.002
Untreated[b]	13 (6)	8x2, 12, 13, 15x3, 16x2, 18, 22, 23x2	15.7 ± 1.4	15	<0.001

* Denotes nehrectomy

a Wilcoxon rank sum test

b Data presented from Table 1

(PNF) animals with primary non-function of their islet graft and not returning to normoglycemia (>9.4 mmol/L) within 2 to 3 days post-transplant and therefore not included in the study.

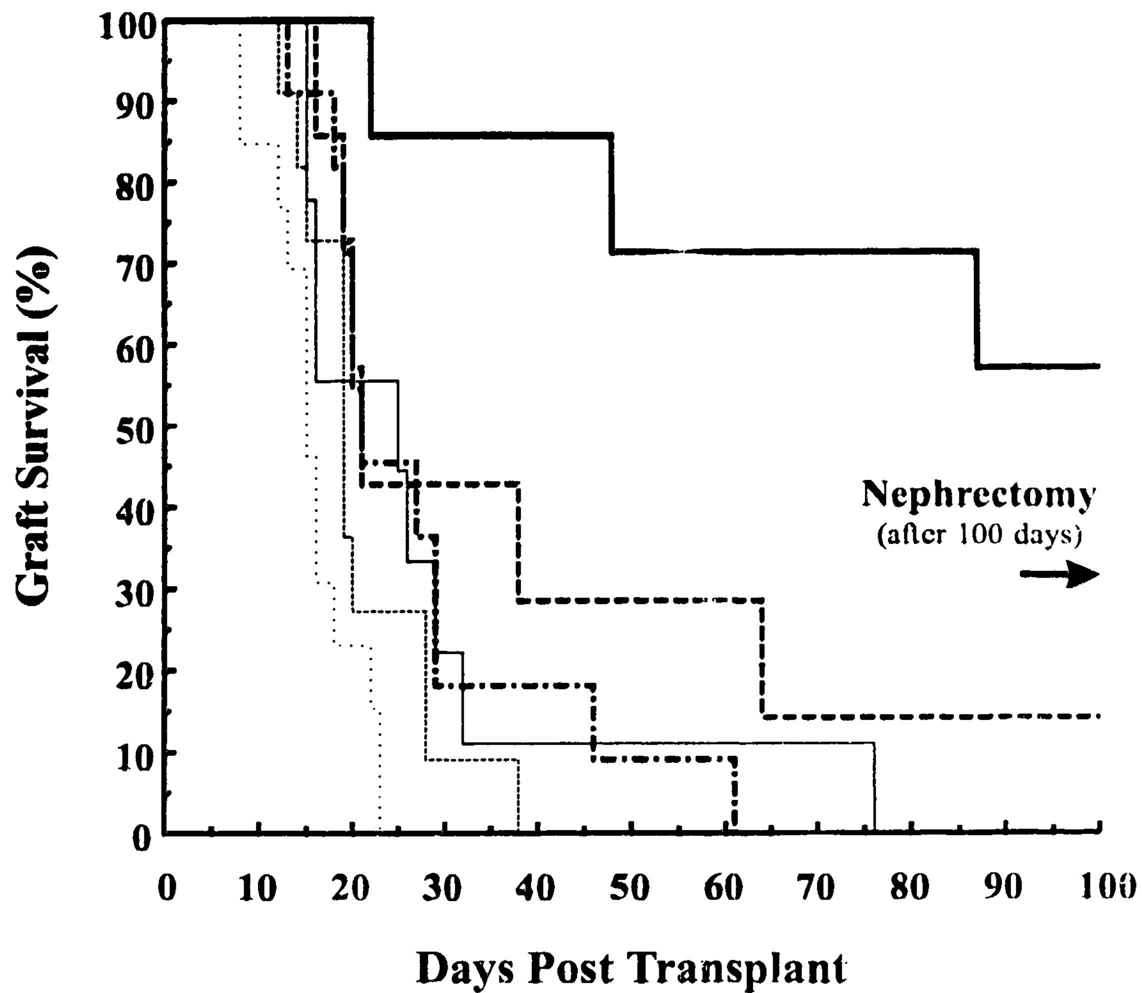


Figure 5. ***Time course of the prevention of recurrence of diabetes in NOD mice receiving islet transplants and heat shock proteins (hsp).*** Female mice receiving injections of hsp65kDa (*M. leprae*) (n=7)(——) or hsp65kDa (*M. bovis*) (n=7) (- - -) or hsp70kDa (*M. tuberculosis*) (n=11) (–•–) in the hind footpads at the time of transplantation; mice receiving injections of IFA (—) (n=9) or saline (n=11)(······) in the hind footpads at the time of transplantation; mice receiving islets without footpad injections (n=13)(· · ·).

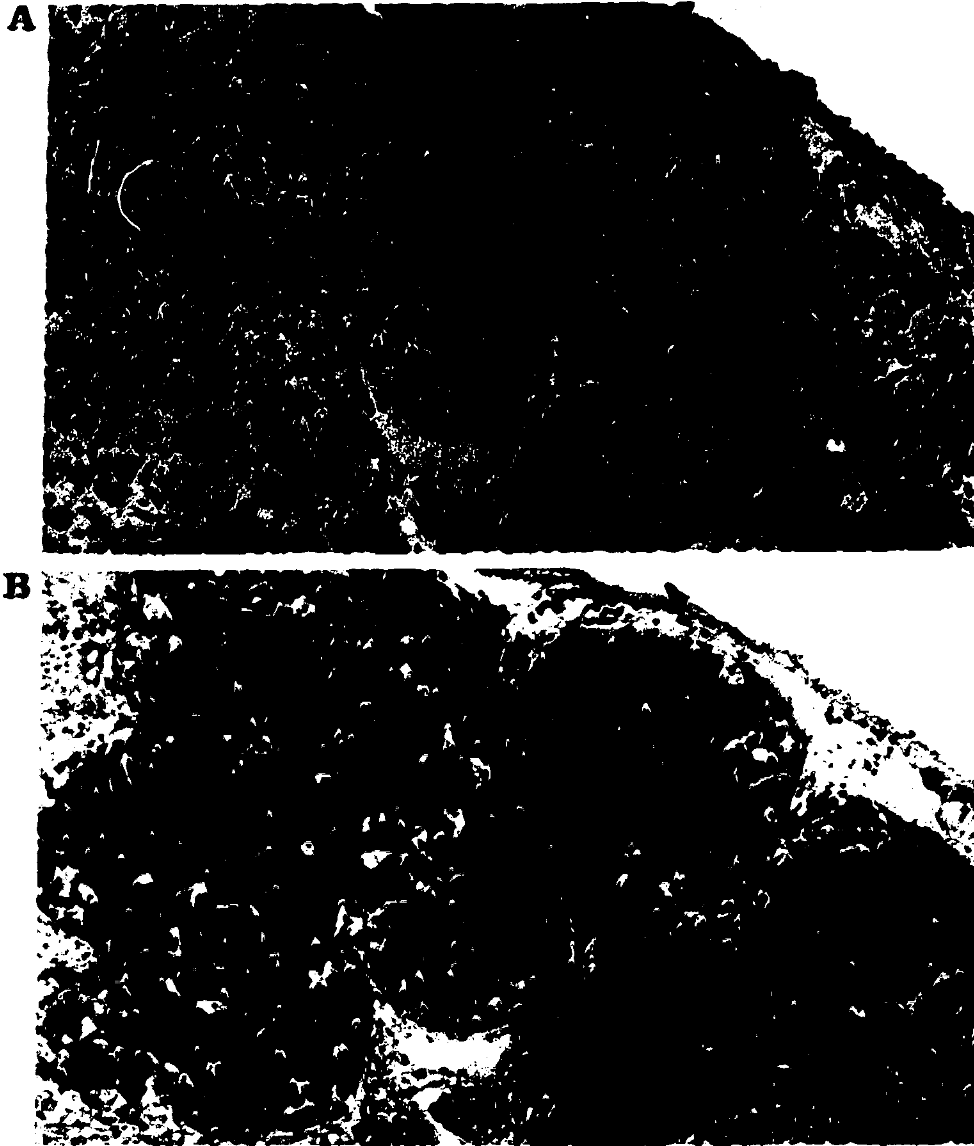


Plate XIV: Photomicrographs of syngeneic islet grafts transplanted under the renal capsule of a diabetic NOD mouse that received 50 μg of *Mycobacterium leprae* derived heat shock protein (hsp-Ml) and remained normoglycaemic until receiving a nephrectomy at 138 days post-transplant. **(A)** hematoxylin-eosin stained section showing several large and small islets surrounded but not infiltrated by mononuclear cells. **(B)** aldehyde-fuchsin stained section showing well-granulated beta-cells as indicated by the dark stained areas (original magnification x 200).

IV
DISCUSSION

Nonspecific immune intervention in a young prediabetic NOD mouse has been reported to decrease the incidence of diabetes. Administration of microbial agents such as BCG, CFA, *Mycobacterium tuberculosis* heat shock protein, or specific immune stimulation with a ß-cell autoantigen, glutamic acid decarboxylase (GAD), can prevent the development of insulitis, ß-cell destruction and IDDM in NOD mice (130,131,147,148,153,154,171). As well, treatment with CFA or BCG at the time of syngeneic islet transplantation prevents islet ß-cell destruction and disease recurrence (92,166,167). The underlying mechanisms of the autoimmune response to islet ß-cell destruction in the spontaneous onset of diabetes and the recurrence of diabetes post-transplant in the NOD mouse are not yet defined however, it is hoped that investigation of the role of CFA and BCG in these pathways will further our understanding in the pathogenesis of IDDM.

The success of immune intervention to prevent the onset of diabetes in the NOD mouse has contributed to the development of clinical trials designed to halt the course of disease in newly diagnosed diabetic patients. The natural course of ß-cell destruction can be altered clinically with agents such as CsA and azathioprine although the majority of successful cases report an extension of the honeymoon period and a return to hyperglycaemia after treatment is discontinued (46,47,170). The lack of prolonged remission of

diabetes in these patients can be attributed to the initiation of therapy too late in the course of disease when there is little or no residual ß-cell function. Because these immune intervention strategies are difficult to study in human patients that have diabetes or are at a high risk of developing the disease, the NOD mouse is an excellent model of IDDM for studying therapies with clinical relevance for human subjects who are predisposed to developing diabetes. The pancreas of a prediabetic NOD mouse is characterized by selective destruction of the ß-cells by the autoreactive T-cells. These animals develop clinical symptoms of diabetes: insulin deficiency, hyperglycaemia, ketosis and overt diabetes (97), all symptoms that are present in IDDM patients. Due to the fact that the majority of clinical intervention strategies are presently restricted to newly diagnosed diabetic patients who have minimal ß-cell function, islet transplantation into diabetic NOD mice is a better model to further our understanding of immune intervention strategies for prevention of the disease itself.

An attractive alternative to daily insulin therapy for a diabetic patient is endocrine replacement of the defective cells (25-28). This form of therapy faces two major immunological problems, allograft rejection and the disease recurrence as a result of the autoimmune destruction of the transplanted islets. In clinical and experimental settings, treatment of the allograft response to the transplanted tissue has been accomplished with immunosuppressive agents such as prednisone, azathioprine and CsA (25-28). The requirement for continuous use and the late stage complications due to over-

immunosuppression make therapy with immunosuppressive drugs an unfavourable alternative over daily insulin therapy. One option shown to work well in murine models of IDDM is to reduce the risk of allograft rejection by decreasing the immunogenicity of the islets with *in vitro* culture prior to transplantation. Despite promising results in rodents, the potential for graft failure due to recurrent autoimmune diabetes is still a barrier to successful allograft transplantation. Wang *et al* reported a study of allograft islet transplantation in the NOD mouse where the immunogenicity of the allogeneic tissue had been reduced with *in vitro* culture (94). The MHC-incompatible (allogeneic) transplanted islets in this study were destroyed by the autoimmune disease process indicating that the T-cell responsible for disease recurrence in the allograft islets was not restricted by the MHC antigens present on the target islet tissue. This report suggests that the recurrence of diabetes in clinical islet transplantation may be a more difficult barrier to overcome than the allograft response to the transplanted tissue which can be alleviated by *in vitro* culture. Further study of the disease recurrence of diabetes in allogeneic and syngeneic tissue transplanted in the NOD mouse is required before widespread clinical application of islet transplantation for the patients already diagnosed with type 1 diabetes can begin.

EVALUATION OF ADJUVANT THERAPY

As mentioned, it has been demonstrated that adjuvant therapy with CFA is effective in preventing ß-cell destruction and the onset of

diabetes in both the spontaneous model and islet transplant model in NOD mice (130,131,166). The importance of this work is that the autoimmune disease was present at the time of transplant and administration of the mycobacterial extracts appeared to suppress the autoimmune process or induce a state of tolerance to the transplanted tissue. This has been confirmed in Group 1 where CFA prevented autoimmune destruction of the transplanted islets, thus substantiating the previous work of Wang and Uleato (92,166). Previous work however never included a group of animals treated with IFA which is the oil component of CFA. In this study an IFA group is included to eliminate the possibility that IFA was not responsible for the protective effect of CFA. In Group 1, animals administered with CFA at the time of islet transplantation showed extended graft survival with 60% of the animals going long term. The majority of animals administered with IFA rejected their grafts by 32 days with only one animal going to 76 days before the onset of hyperglycaemia, a range of survival much lower than the CFA group. This finding is in agreement with Qin who found that a single injection of IFA in 5-week-old NOD mice did not prevent the onset of diabetes (133). This result cannot be directly compared with our study, however, it does serve to validate our findings of the lack of protective effect exhibited by IFA.

In another study, Lakey *et al* reported extended graft survival using another immunoadjuvant live BCG vaccine (167). It is not clear what component(s) of the *Mycobacteria* present in CFA or BCG is responsible for protecting the islets from autoimmune attack post-

transplantation. The major difference between the two adjuvants is that *M. tuberculosis* present in CFA is killed whereas *M. bovis* constituting the BCG vaccine is live. CFA administered at a dose of 50 μg has been reported to prevent the onset of the disease as well as protecting the transplanted islets from autoimmune destruction (92,130,131,166). CFA is not approved for humans therefore a more clinically relevant adjuvant is needed. BCG is used clinically and has been shown to prevent the spontaneous onset of diabetes when administered at a dose of 1.0 mg (130) as well as protecting transplanted islets when 2.7 mg is administered at the time of transplantation (167). Unpublished results from Lakey et al in a dose-related study of live BCG vaccine indicated that unless BCG is administered at this high dose, complete protection of islet grafts past 100 days is not possible. The dose of 2.7 mg/mouse is unrealistic for clinical application, therefore, it is important to isolate the protective component of BCG that would allow a use for humans.

It was hypothesized that a 50 μg concentration of heat killed *M. bovis*, which would be more applicable for clinical use than CFA should have a protective effect on the transplanted islets. The results in Group 3 conclude that the therapeutic effect of dead *M. tuberculosis* in CFA to protect transplanted islets is not provided by dead *M. bovis* which was administered to a transplant recipient at the same dose as CFA, 50 μg per mouse. The difference between the range of graft survival of the CFA group and the 50 μg *M. bovis* group (Tables 1 and 3) and the percentage of animals having graft survival past 100 days confirms that treatment with CFA lends to

substantially more animals showing long term graft survival. It was also hypothesized that killed *M. bovis* in an oil emulsion could function at the reported dose of live BCG, 2.7 mg/mouse (167). Killed *M. bovis* administered to transplant recipients at a dose of 2.7 mg per mouse did not protect the transplanted islets (Table 3) as compared to the published results of live BCG at the same dose. These results prove that for *M. bovis* to induce a state of tolerance and thus protect the islets from autoimmune destruction, the bacteria must be live and administered at a dose of 2.7 mg per mouse.

The histological evidence of the accumulation of monocytic and lymphocytic cells (peri-insulitis) surrounding the transplanted islets in CFA protected animals implicates a regulatory function by an unidentified cell population that prevents ß-cell destruction by autoreactive T-cells (145). Many reviews of immune intervention in NOD mice have stated that the protective effects of CFA are the result of increased levels of antigen nonspecific activity *in vitro* typical of natural suppressor cell populations (115-118). A report that substantiates the presence of these cell is the cyclophosphamide-induced diabetes in CFA protected animals that was suggested to have occurred by the deletion of the regulatory T-cells by the administration of cyclophosphamide which in turn activated the T-cell dependent ß-cell destruction (133). This report and the evidence of the protective effect against diabetes provided by CFA points to the activation of a regulatory T-cell population as induced by CFA that prevents ß-cell destruction in the spontaneous and disease recurrence models of diabetes in NOD mice. The other immunoadjuvant that

exhibits a protective effect on transplanted islets is BCG vaccine which is also reported in the literature for its suppression of humoral and cell mediated immunity (143,144, 167,173,174). The suppressive effect of BCG in animals protected from the spontaneous disease has been identified by one group of investigators to be the generation of suppressor macrophages which inhibit lymphocyte functions (173,174). A recent study by Lakey et al confirm the presence of a regulatory cell in BCG that protected the transplanted islets where the autoimmune destruction of the graft was initiated after administration of cyclophosphamide to animals with long term acceptance of their islet grafts (167,168). The regulatory mechanism and phenotype of the cell(s) that account for these results are not yet defined (work of Dr. T. Halstenson in progress). The results presented in this thesis do not distinguish between which population of cells are responsible for the protective effects of adjuvant therapy with CFA and BCG. The results do serve to delineate which *Mycobacterium* and in what conformation (live or heat killed) is responsible for the protection of transplanted islets from the T-cell mediated destruction. The protective effect can be attributed to *M. tuberculosis* cell wall components and a large dose of live BCG vaccine, and can not be attributed to the oil component of CFA, or to a prepar[illegible] heat killed *M. bovis* (BCG).

GAD AND DISEASE RECURRENCE

The 64kD autoantigen glutamic acid decarboxylase (GAD), as identified by Baekkeskov in 1990, has been implicated in the presence

of GAD reactive T-cells and antibodies in preclinical and recent-onset diabetics (43). With the discovery of GAD reactive T-cells in 4-week-old NOD mice and the ability to tolerize against this autoantigen, it is expected that the precise role of GAD in the pathogenesis of IDDM will be identified very soon (147,148,171). In this study we found no role for GAD in preventing disease recurrence post-islet transplantation. The isoforms of GAD administered to islet recipients were neither able to tolerize nor prevent the autoreactive T-cells from destroying the transplanted syngeneic islets. It is therefore hypothesized that the autoreactive T-cells were not tolerized to the autoantigen GAD after the natural course of diabetes was in place. Because of this non-tolerizing effect, disease recurred in the transplanted islets after treatment with 50 µg of mouse GAD67 or human GAD65 in 70% of the animals. It is interesting to note that in each of the GAD67 and GAD65 groups approximately 30% of animals did have extended graft survival past 100 days. The control protein, PFSY-A1, which is not a ß-cell autoantigen, also extended graft survival to the same extent as did GAD65 and GAD67. The lack of significant difference between results of the control protein and the GAD treated animals indicates that GAD administered at the time of transplant did not protect the syngeneic islets from autoimmune destruction. Contrary to these results, recent studies have shown that T-cells from young NOD mice can be tolerized against the autoantigen GAD when recombinant GAD65 protein is injected into young NOD mice (147,148). In these studies there was a marked decrease in the incidence of diabetes indicating that GAD is a key

autoantigen in the induction of murine IDDM. The precise mechanism of the disease recurrence of diabetes in transplanted NOD mice is not fully understood and it remains to be seen whether or not GAD has an important role in this model of IDDM.

PROTECTIVE EFFECT OF HEAT SHOCK PROTEINS

Heat shock proteins (hsp) are highly conserved molecules that are also extremely antigenic (175,176). Given their conserved phylogeny, hsp are very universal in nature and hsp responses can be observed in almost every prokaryotic and eukaryotic cell. Hsp also serve as antigens of infectious agents and are therefore involved in immunity. Accordingly, it has been discovered that hsp65, a member of the hsp60 family, is a target for antibodies for a variety of pathogens including *M. leprae* and *M. tuberculosis*. As well, particulars of the hsp70 family have also been implicated as antigens in infections with both *M. leprae* and *M. tuberculosis* (176). The association of hsp65 with the disease rheumatoid arthritis induced in rats with adjuvant therapy suggests a role for this particular hsp in autoimmune disease (175). Rats immunized with hsp65 do not develop arthritis in an experimental setting and in human patients with rheumatoid arthritis, T-cell responses to mycobacterial hsp65 have been noted (175,176). Brudzynski et al have speculated on the role of a 60kD heat shock-related protein in normal ß-cell physiology (178). The observed changes in the cellular distribution of hsp60 within the secretory granules of insulin producing ß-cells corresponded with the progression of insulitis in prediabetic NOD

mice. The change observed is suggested to lead to new antigenic sites on the ß-cell contributing to the development of autoimmunity and the onset of diabetes (178).

Prevention of the spontaneous onset of diabetes by the vaccination with hsp or with hsp specific T-cells in NOD mice has been reported previously (153,154). Elias et al have also noted the high sequence homology of the mouse hsp65 with the human hsp65 molecule. A recombinant human hsp 65 peptide, p277, stimulates T-cell clones in non-diabetic NOD mice. Our preliminary study of the role of hsp in the disease recurrence model of IDDM in NOD mice have suggested a role for the 65kD hsp derived from *M. leprae*. Sixty percent of animals receiving an islet transplant and 50 μg of hsp65 (*M. leprae*) showed extended graft survival past 100 days post-transplant. The protective effect of this hsp became apparent when compared with results obtained by vaccination of mice with hsp from *M. bovis* or *M. tuberculosis* at the time of transplantation which did not prevent the disease recurrence of diabetes. In the spontaneous model of IDDM, the protective effect of hsp is thought to occur through an immunosuppressive effect on the active components of the immune system responsible for ß-cell destruction (153,154). Hypothetically, the protective effect by hsp65 (*M. leprae*) demonstrated in the disease recurrence model of diabetes could also be attributed to a suppressor effect on the active components of the disease. In the case of disease recurrence, the T-cells responsible for the initial ß-cell insult on the islets in the pancreas are already activated and therefore may already be primed to a hsp65 located on

the ß-cell which was identified by Brudzynski (178). The fact that these T-cells were prevented from responding to this autoantigen when it is presented on the target islet tissue suggests that the administration of the *Mycobacterium* hsp65 may have tolerized the autoreactive T-cells before they recognized a hsp65 on the transplanted islets therefore not proceeding with autoreactive destruction of the islets. The precise role of hsp65 in mouse and human IDDM is under speculation and warrants further investigation.

With respect to an overall view of the results reported in this thesis it could be assumed that the disease recurrence in NOD mice transplanted with syngeneic islets is delayed when combined with an immune stimulatory treatment. The immune stimulative property of bacterial adjuvants and heat shock proteins has been documented previously and it is assumed that an interruption in the course of disease through the administration of an immune stimulant would delay the autoimmune process of the T-cell reactivity to the transplanted islets. In all cases reported in this thesis, the mean graft survival of groups receiving treatments was significantly longer than the mean graft survival of the untreated group of animals. This delay in disease recurrence however, does not explain the protective effects of CFA and hsp65 (*M. leprae*) which appear to interrupt the disease indefinitely when compared with the various other treatments offered to the NOD mice at the time of syngeneic islet transplant. It can be hypothesized that a very specific component of each treatment targets a yet unknown pathway or pathways in the pathogenesis of diabetes

and suppresses the disease from recurring in transplanted syngeneic islets.

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Appendix A: Ficoll Gradient for Mouse Islet Isolation

Stock Solution: Dissolve 60.0 gr of Ficoll 400 DL (Sigma) in 180 ml Hank's balanced salt solution (HBSS) (Gibco) that has been supplemented with 25 mM HEPES (Gibco).

Ficoll Concentration	Stock	HBSS
25%	60.0 ml	-------
23%	55.2 ml	4.8 ml
21.5%	51.6 ml	8.4 ml
11%	26.4 ml	33.6 ml

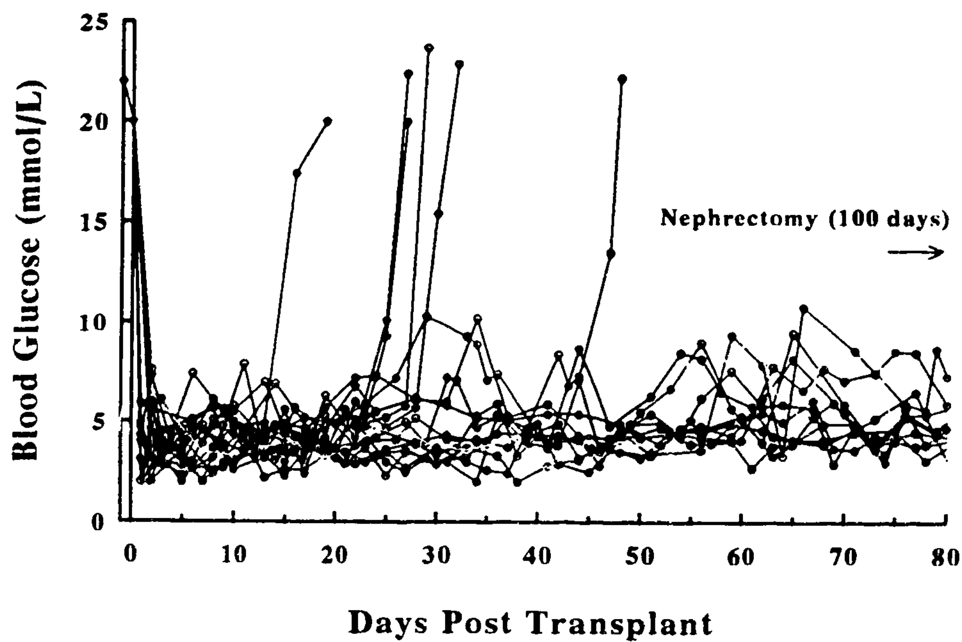

Figure B-1: Blood glucose profiles of individual diabetic NOD mice (n=17) transplanted with syngeneic islets and receiving 50 μg of complete Freund's adjuvant at the time of transplantation. The range of graft survival in this group was from 16 days with 11 mice showing long term graft survival and receiving nephrectomies.

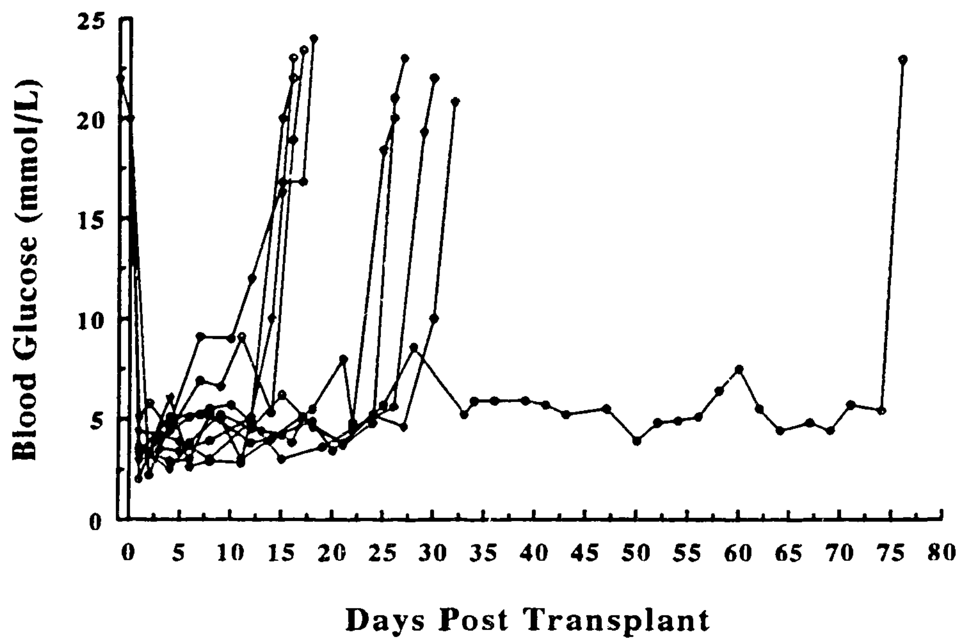

Figure B-2: Blood glucose profiles of individual diabetic NOD mice (n=9) transplanted with syngeneic islets and receiving incomplete Freund's adjuvant at the time of transplantation. The range of graft survival in this group was from 15 to 76 days before rejection (recurrence of hyperglycemia).

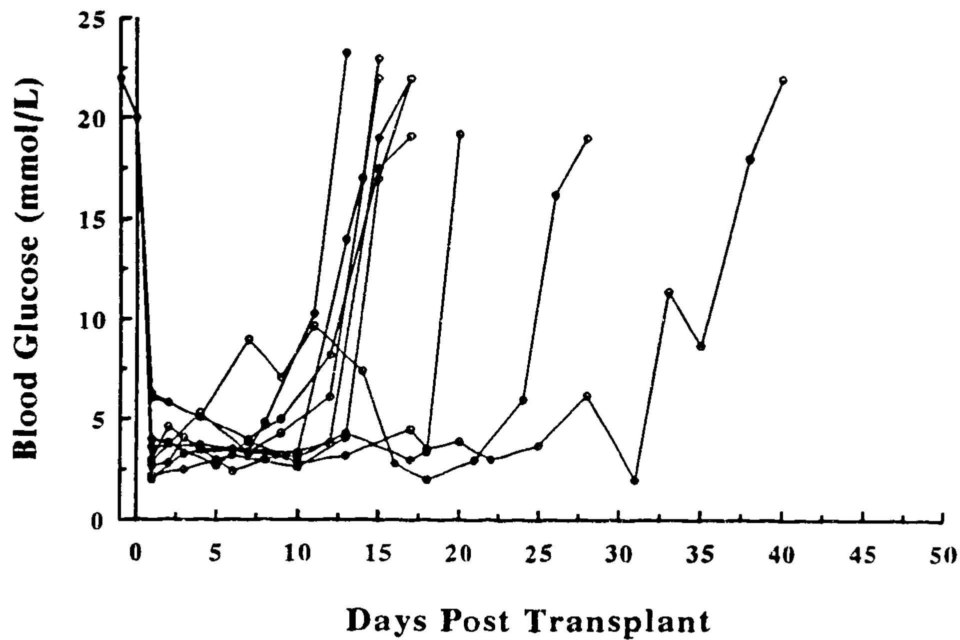

Figure B-3: Blood glucose profiles of individual diabetic NOD mice (n=11) transplanted with syngeneic islets and receiving saline at the time of transplantation. The range of graft survival in this group was from 12 days to 38 days before rejection (recurrence of hyperglycemia).

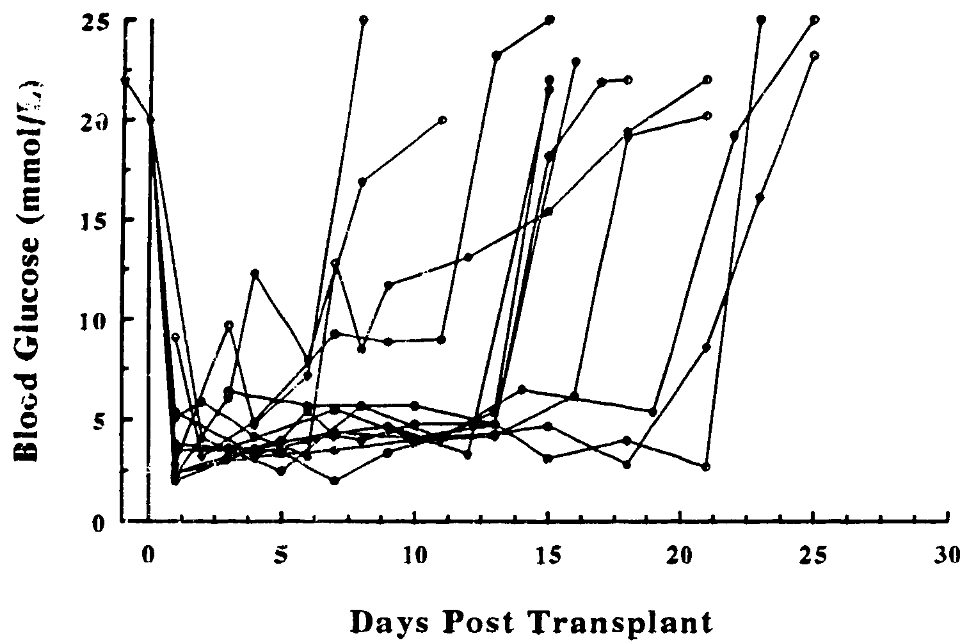

Figure B-4: Blood glucose profiles of individual diabetic NOD mice (n=13) transplanted with syngeneic islets and not receiving a treatment. The range of graft survival in this group was from 8 days to 23 days before rejection (recurrence of hyperglycemia).

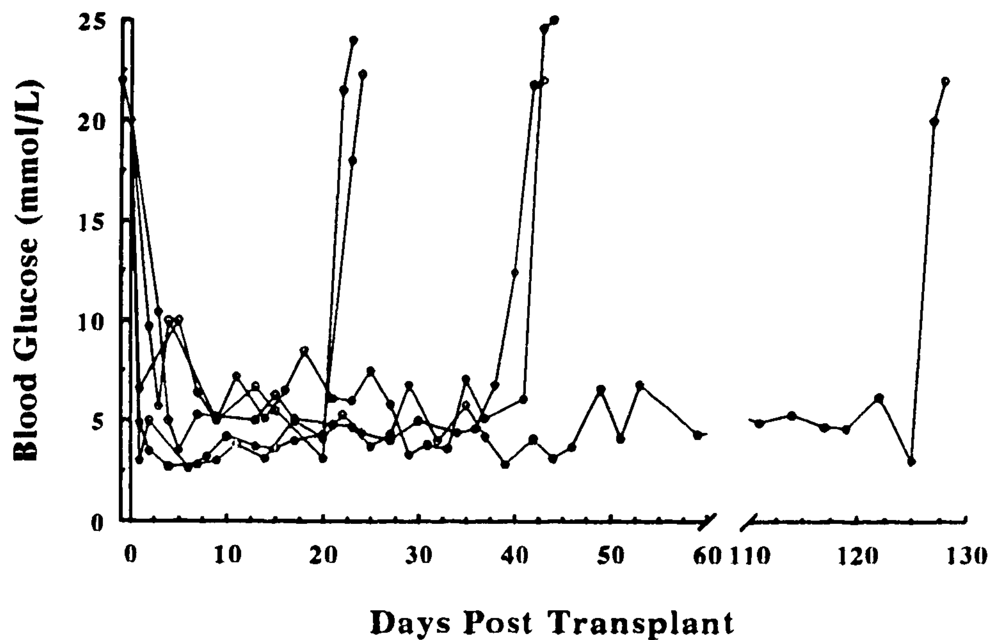

Figure C-1: Blood glucose profiles of individual diabetic NOD mice (n=5) transplanted with syngeneic islets and receiving 100 µg of dead *Mycobacterium bovis* bacteria at the time of transplantation. The range of graft survival in this group was from 22 days to 125 days before rejection (recurrence of hyperglycemia).

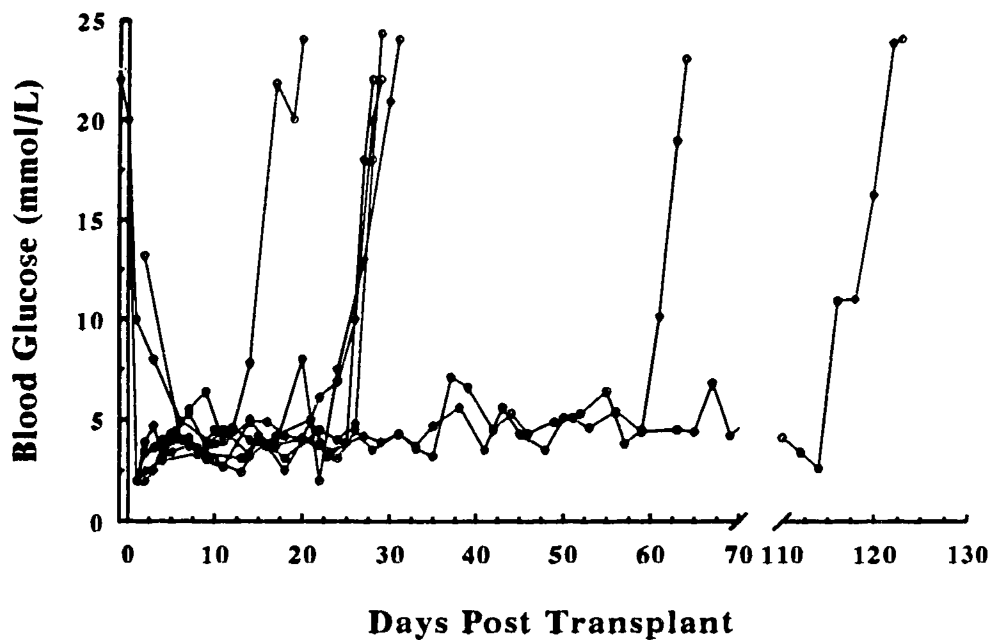

Figure C-2: Blood glucose profiles of individual diabetic NOD mice (n=7) transplanted with syngeneic islets and receiving 100 μg of dead *Mycobacterium bovis* lysate at the time of transplantation. The range of graft survival in this group was from 17 days to 122 days before rejection (recurrence of hyperglycemia).

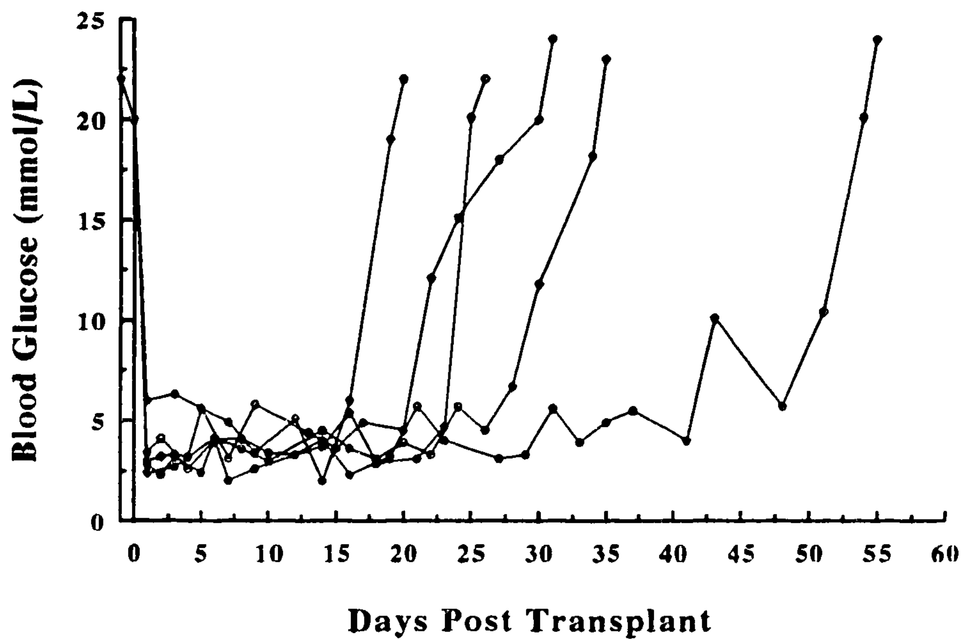

Figure C-3: Blood glucose profiles of individual diabetic NOD mice (n=5) transplanted with syngeneic islets and receiving 100 μg of dead *Escherichia coli* bacteria at the time of transplantation. The range of graft survival in this group was from 19 days to 54 days before rejection (recurrence of hyperglycemia).

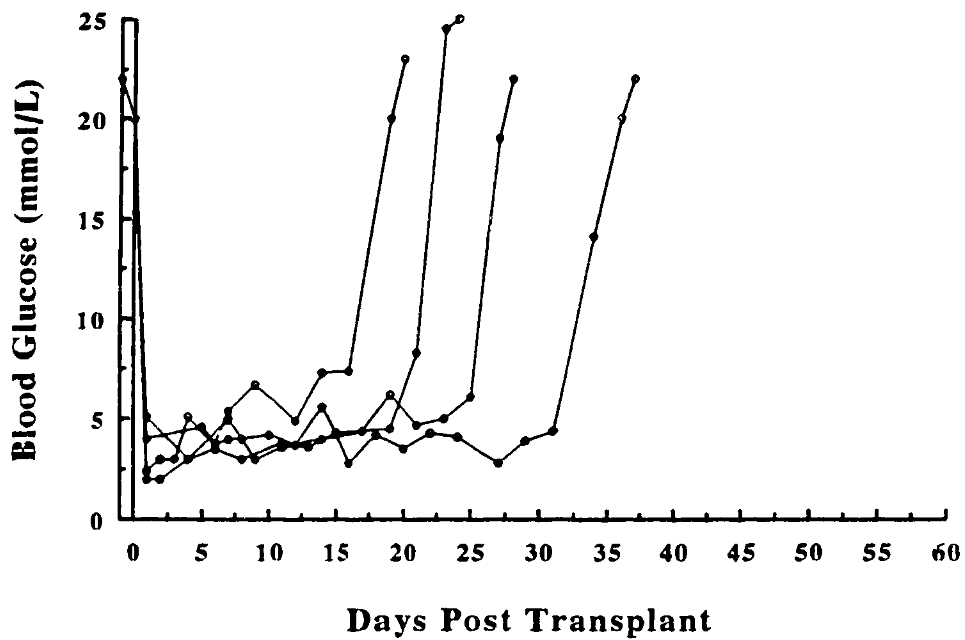

Figure C-4: Blood glucose profiles of individual diabetic NOD mice (n=4) transplanted with syngeneic islets and receiving 100 μg of dead *Escherichia coli* lysate at the time of transplantation. The range of graft survival in this group was from 19 days to 36 days before rejection (recurrence of hyperglycemia).

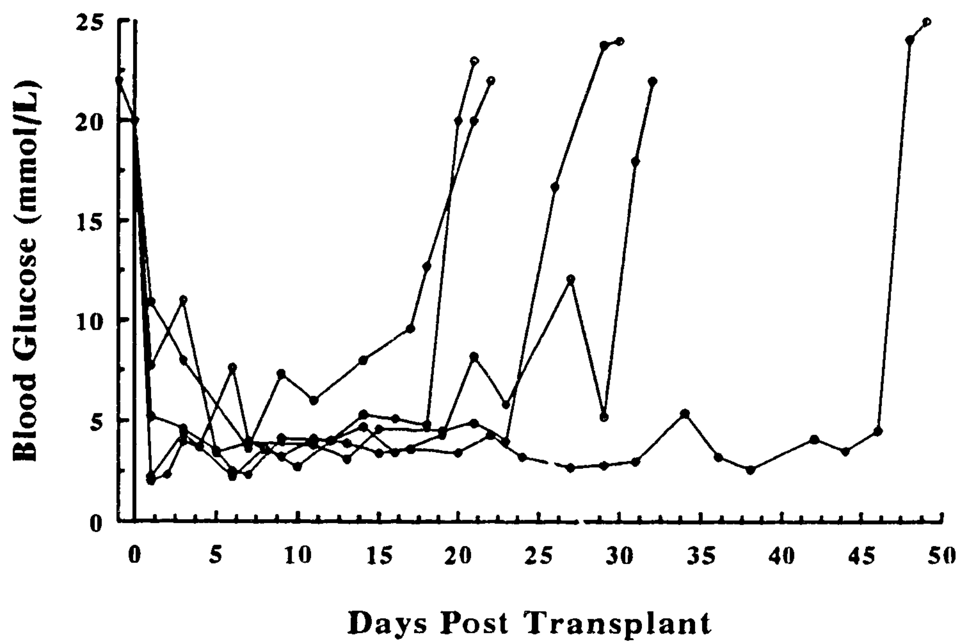

Figure C-5: Blood glucose profiles of individual diabetic NOD mice (n=5) transplanted with syngeneic islets and receiving 100 µg of dead *Salmonella typhimurium* bacteria at the time of transplantation. The range of graft survival in this group was from 20 days to 48 days before rejection (recurrence of hyperglycemia).

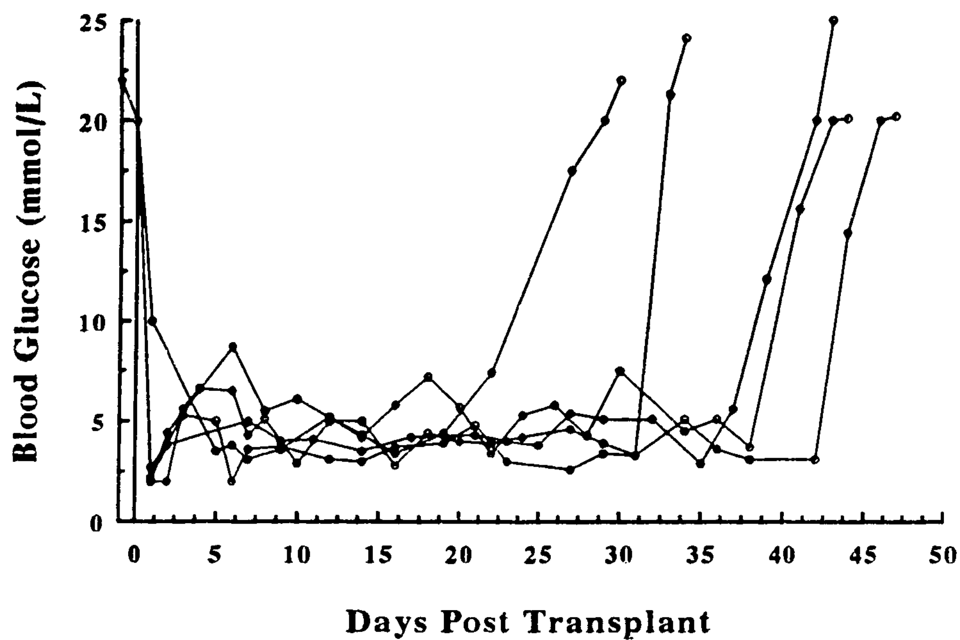

Figure C-6: Blood glucose profiles of individual diabetic NOD mice (n=5) transplanted with syngeneic islets and receiving 100 μg of dead *Salmonella typhimurium* lysate at the time of transplantation. The range of graft survival in this group was from 28 days to 46 days before rejection (recurrence of hyperglycemia).

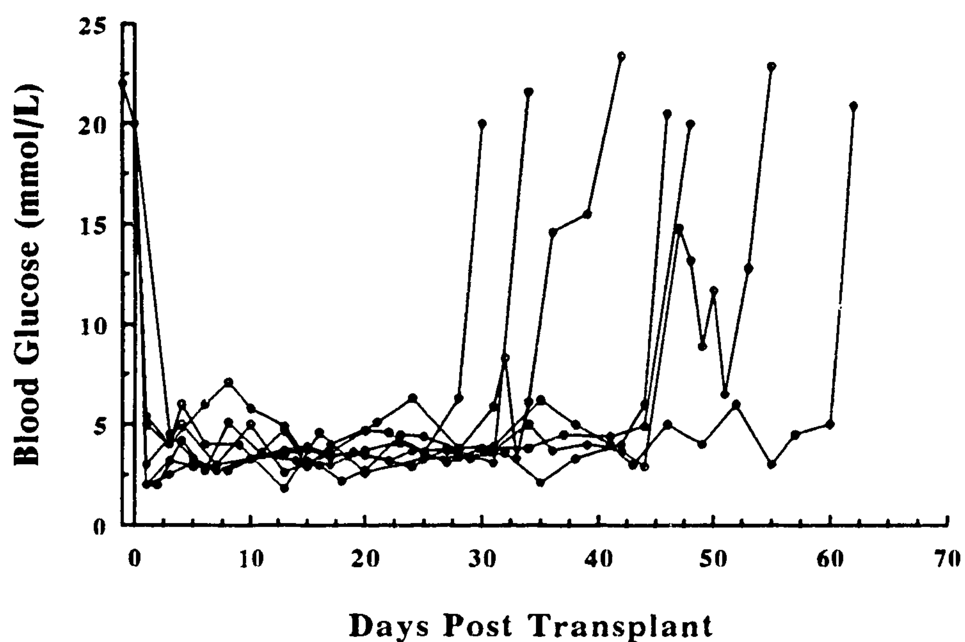

Figure D-1: Blood glucose profiles of individual diabetic NOD mice (n=7) transplanted with syngeneic islets and receiving 2.7 mg of dead *Mycobacterium bovis* at the time of transplantation. The range of graft survival in this group was from 30 days to 62 days before rejection (recurrence of hyperglycemia).

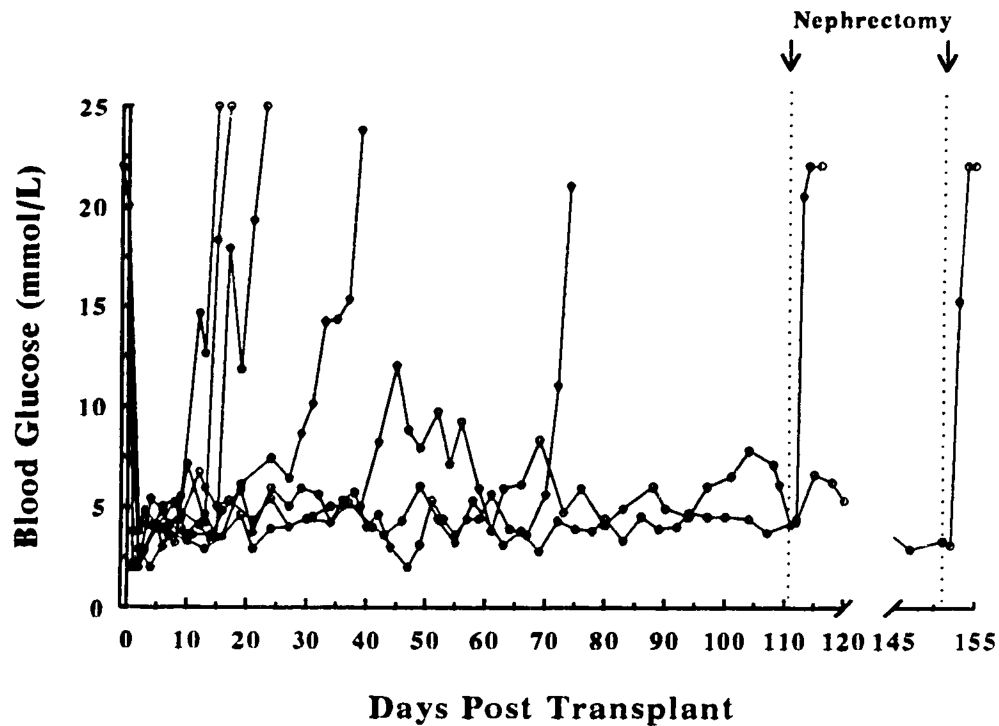

Figure D-2: Blood glucose profiles of individual diabetic NOD mice (n=7) transplanted with syngeneic islets and receiving 50 µg of dead *Mycobacterium bovis* at the time of transplantation. The range of graft survival in this group was from 15 days with 2 mice having graft survival past 100 days and receiving a nephrectomy.

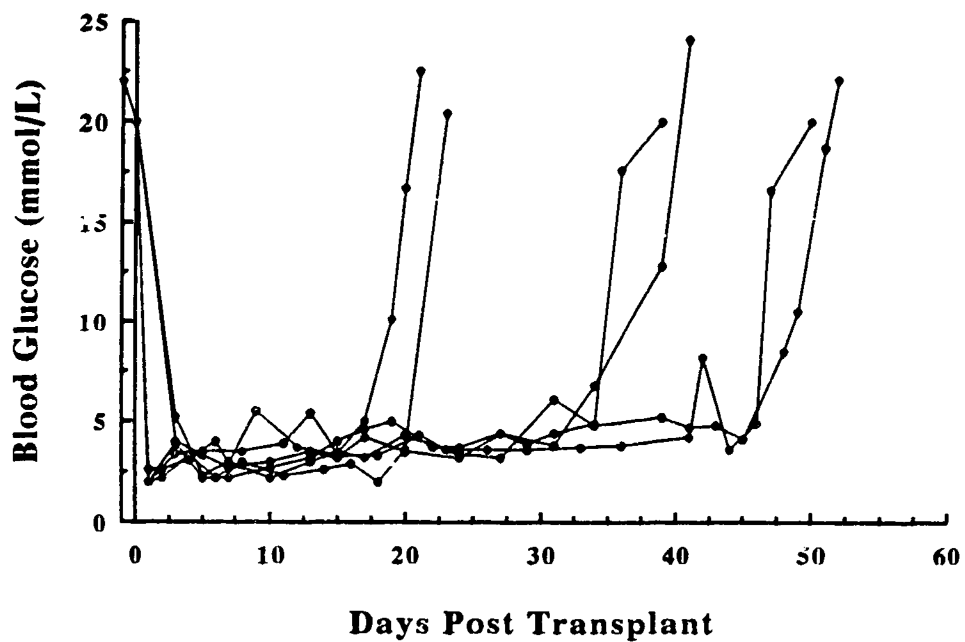

Figure D-3: Blood glucose profiles of individual diabetic NOD mice (n=6) transplanted with syngeneic islets and receiving 2.7 mg of dead *Escherichia coli* at the time of transplantation. The range of graft survival in this group was from 20 days to 55 days before rejection (recurrence of hyperglycemia).

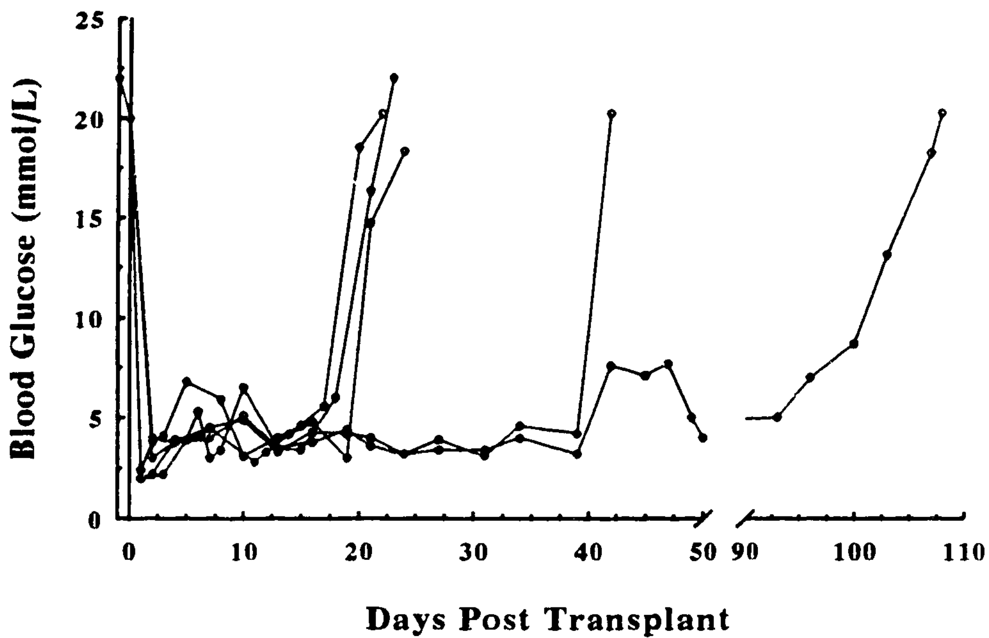

Figure D-4: Blood glucose profiles of individual diabetic NOD mice (n=6) transplanted with syngeneic islets and receiving 50 μg of dead *Escherichia coli* at the time of transplantation. The range of graft survival in this group was from 20 days to 108 days before rejection (recurrence of hyperglycemia).

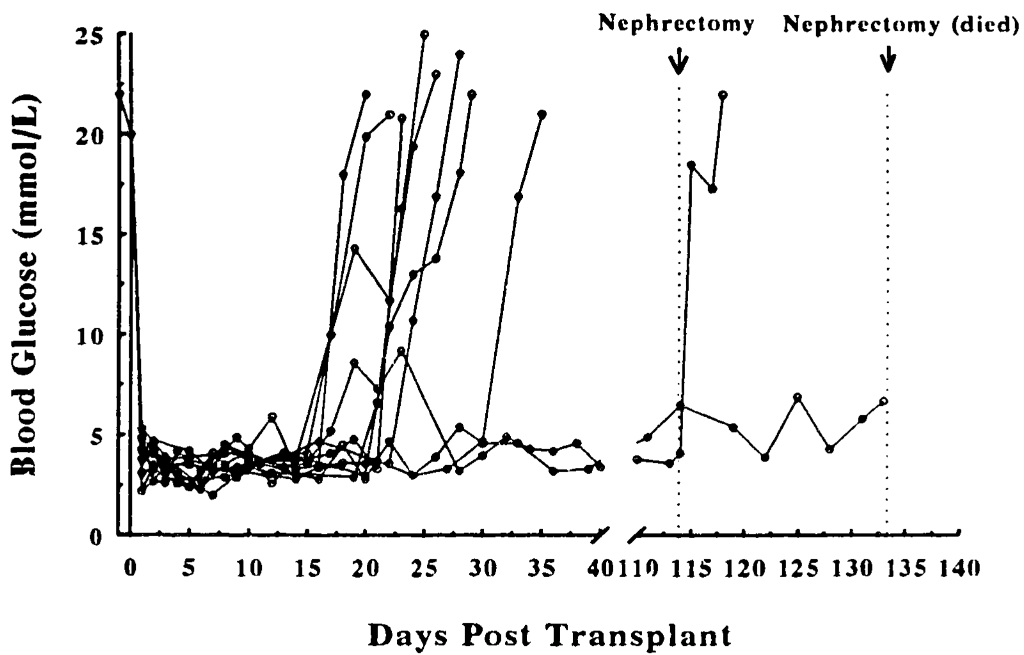

Figure E-1: Blood glucose profiles of individual diabetic NOD mice (n=10) transplanted with syngeneic islets and receiving 50 µg of mouse GAD67 protein at the time of transplantation. The range of graft survival in this group was from 20 days to 2 animals showing graft survival past 100 days and receiving nephrectomies.

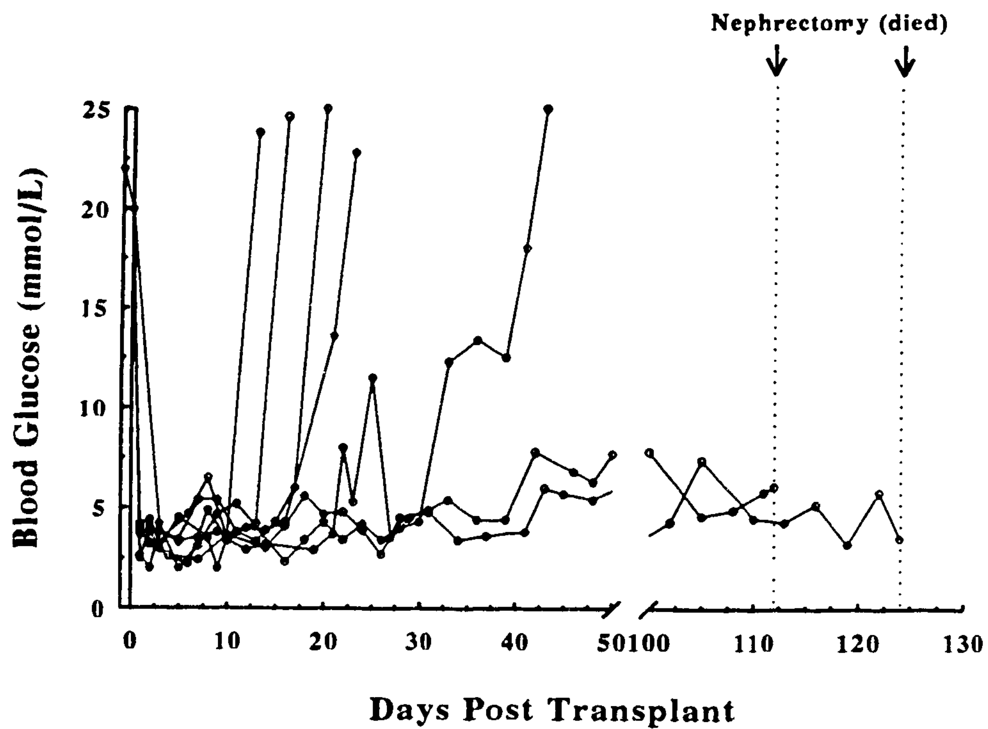

Figure E-2: Blood glucose profiles of individual diabetic NOD mice (n=7) transplanted with syngeneic islets and receiving 50 μg of human GAD65 peptide at the time of transplantation. The range of graft survival in this group was from 13 days to 2 animals showing graft survival past 100 days and receiving nephrectomies.

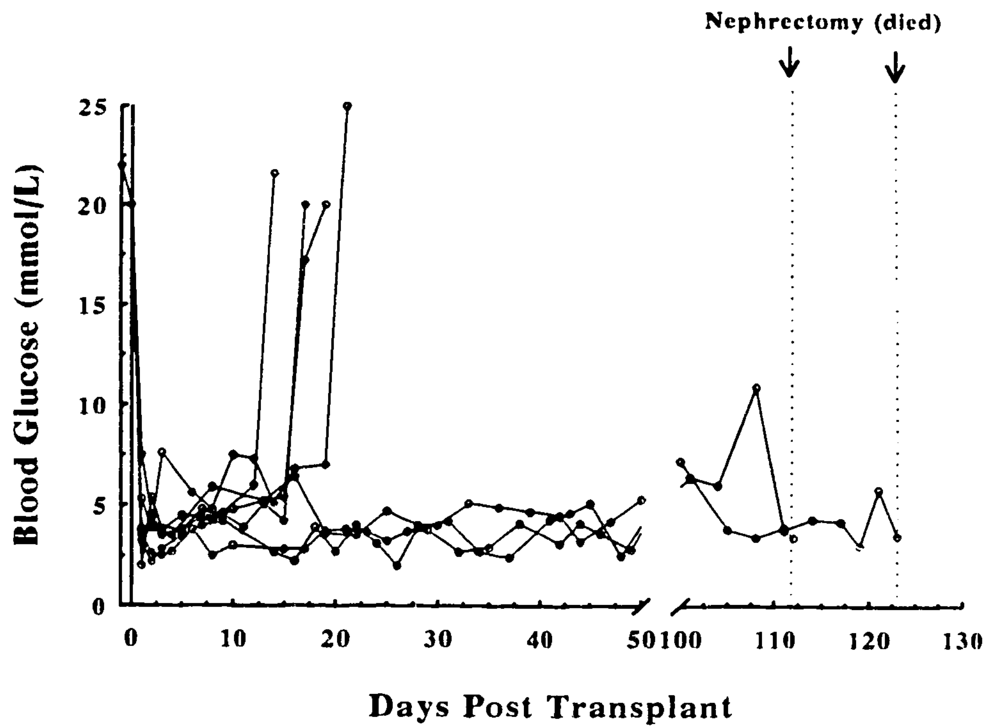

Figure E-3: Blood glucose profiles of individual diabetic NOD mice (n=7) transplanted with syngeneic islets and receiving 50 μg of PFSY-A1 control protein at the time of transplantation. The range of graft survival in this group was from 14 days to 2 animals showing graft survival past 100 days and receiving nephrectomies.

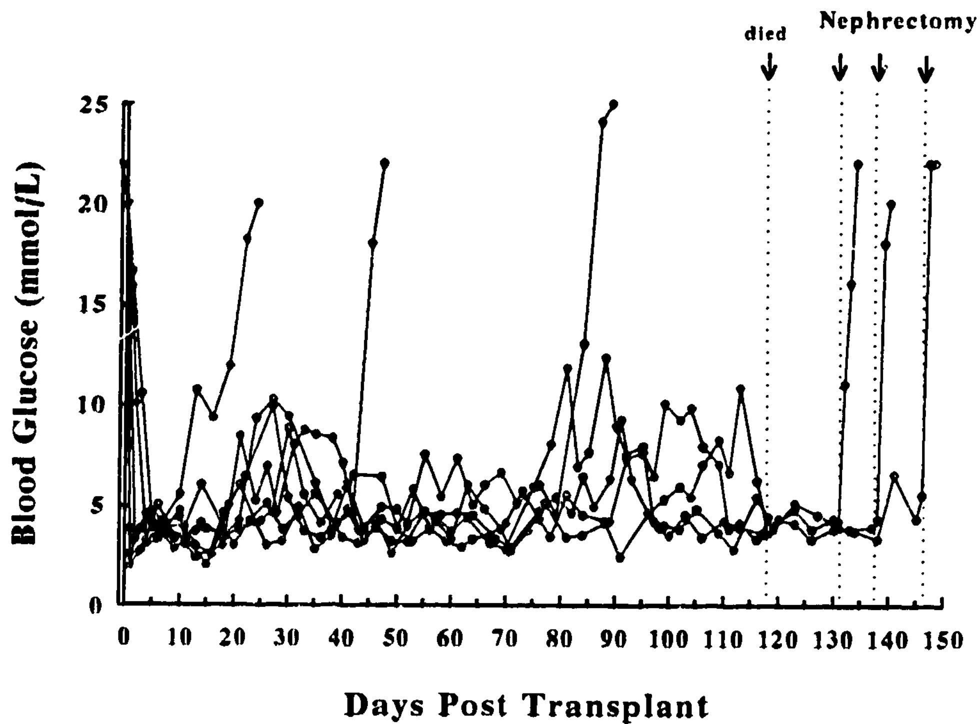

Figure F-1: Blood glucose profiles of individual diabetic NOD mice (n=7) transplanted with syngeneic islets and receiving 50 μg of *Mycobacterium leprae* derived heat shock protein (65kD) at the time of transplantation. The range of graft survival in this group was from 22 days with 4 animals having graft survival past 100 days and receiving a nephrectomies.

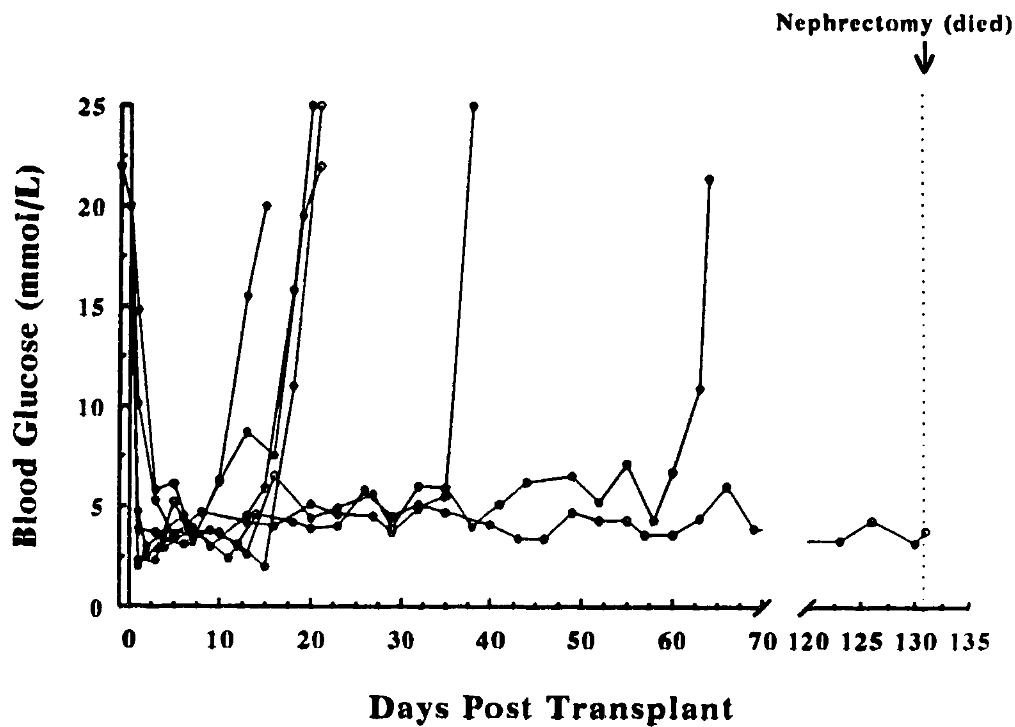

Figure F-2: Blood glucose profiles of individual diabetic NOD mice (n=7) transplanted with syngeneic islets and receiving 50 μg of *Mycobacterium bovis* derived heat shock protein (65kD) at the time of transplantation. The range of graft survival in this group was from 16 days with 1 animal having graft survival past 100 days and receiving a nephrectomy.

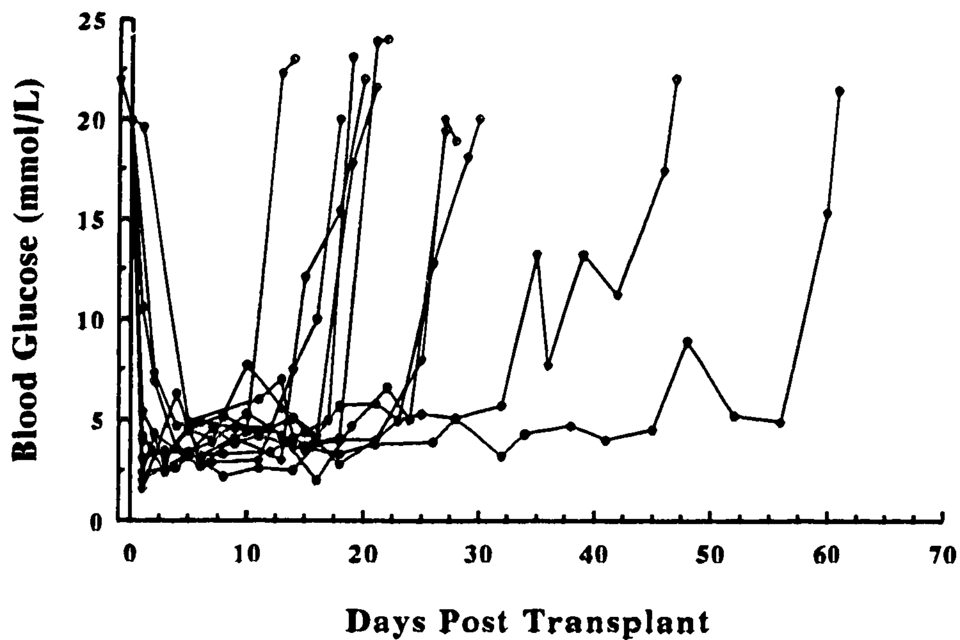

Figure F-3: Blood glucose profiles of individual diabetic NOD mice (n=7) transplanted with syngeneic islets and receiving 50 μg of *Mycobacterium tuberculosis* derived heat shock protein (70kD) at the time of transplantation. The range of graft survival in this group was from 13 days to 61 days before rejection (recurrence of hyperglycemia).