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THE UNIVERSITY OF ALBERTA

CRYOPRESERVATION AND IMMUNO-ALTERATION OF TRANSPLANTED RAT ISLETS OF LANGERHANS

BY

MARILYNE G. COULOMBE

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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October 8, 1987

Ms. Marilyne Coulombe
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Dear Ms. Coulombe:

Permission is hereby granted to use "The reversal of diabetes by transplantation of cryopreserved rat islets of Langerhans to the renal subcapsular space" and "Prolongation of islet xenograft survival by cryopreservation" as part of your M.Sc. thesis.

Sincerely,

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submitted by **MARILYNE G. COULOMBE**

in partial fulfilment of the requirements for the degree of **MASTER OF SCIENCE in EXPERIMENTAL MEDICINE.**

Supervisor

Date: 05, 1987

This text is dedicated to

GUY

my best friend

my constant source of support and encouragement

ABSTRACT

Pancreatic islet transplantation has attracted much interest as an alternative to exogenous insulin therapy for the treatment of insulin-dependent diabetes mellitus. This technique can reverse experimentally induced diabetes in rodents and larger animals and has been shown to prevent the debilitating complications of the disease. Many advances have been made in (i) obtaining, from a single pancreas, quantities of islet tissue sufficient to normalize one recipient, (ii) reducing immunogenicity of islet tissue to avoid the need for long-term immunosuppression, and (iii) preserving islet tissue for transportation between transplant centers or to allow time for the preparation of a suitable recipient. Cryopreservation of islet tissue would greatly facilitate clinical application of islet transplantation in each of these areas.

Rodent islets, isolated by collagenase digestion and Ficoll purification, can be successfully cryopreserved by slow cooling to -40°C and rapid thawing. This was demonstrated by implanting 3000 fresh or frozen-thawed rat islets beneath the kidney capsule of streptozotocin-induced diabetic rats. Plasma glucose, urine volume, urine glucose and weight returned to normal values after transplantation and removal of the graft-bearing kidney resulted in an immediate return to the diabetic state. No differences were observed between groups after one week post-implant or in glucose tolerance tests performed four months after transplantation. This is conclusive evidence for islet viability following cryopreservation.

Many studies have shown that pretreating rodent islets with culture, ultraviolet irradiation, or Ia-antibody can result in

prolonged or indefinite islet allo- or xenograft survival. These approaches to reducing islet immunogenicity selectively destroy or inactivate immunostimulatory leukocytes within the islet. Because distinct cell types are differentially susceptible to a particular freeze-thaw process, islet immunogenicity could be modulated with a cryobiological approach. This possibility was examined in rodent allo- and xenograft models.

Implanting 500 rat islets beneath the kidney capsule of diabetic mice normalized clinical indices within two days. By 13 days post-transplant, all fresh xenografts had rejected, whereas 38% of cryopreserved grafts were still functioning. When anti-lymphocyte serum was given at the time of transplantation, 6% of fresh xenografts and 55% of frozen-thawed grafts were functioning 19 days after implantation. These results show that cryopreservation can reduce islet immunogenicity. A protocol which would maximize damage to immunostimulatory leukocytes without sacrificing islet viability would be invaluable to the clinical application of islet transplantation.

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ALS	anti-lymphocyte serum
APC	antigen-presenting cell
CyA	cyclosporin-A
DMSO	dimethyl sulfoxide
ivGTT	intravenous glucose tolerance test
LEW	Lewis strain of rat ($RT1^l$)
MALS	rabbit anti-mouse lymphocyte serum
MHC	major histocompatibility complex
MLIC	mixed lymphocyte islet culture
PG	plasma glucose (mg/dL)
RALS	rabbit anti-rat lymphocyte serum
TLI	total lymphoid irradiation
UG	urine glucose (g/24h)
UV	urine volume (mL/24h)
UVB	ultraviolet irradiation
WF	Wistar-Furth strain of rat ($RT1^u$)
WT	weight (g)

INTRODUCTION

Prior to the 1921 discovery of insulin by Banting and Best (1), diabetes mellitus was associated with death within a year of diagnosis. Since that time insulin therapy has greatly increased the life expectancy of diabetic patients, yet, today, diabetes mellitus with its associated complications remains a major medical problem, resulting in high morbidity and mortality and imposing a financial burden on the health care system. In contrast to a nondiabetic population of comparable age, diabetic patients are 25 times more prone to blindness, 17 times more to kidney disease, 16 times more to gangrene and lower-extremity amputations, and at least twice as prone to cardiovascular disease (2). This chronic disease is the leading cause of blindness in North American adults and contributes to one-third of all cases of end-stage renal disease and almost half of all nontraumatic amputations in adults (2,3). Thus, at diagnosis, the life expectancy of the estimated 11 million North Americans with diabetes mellitus is one-third less than that for a person without diabetes (2).

Data from both experimental and clinical studies suggest that diabetic nephropathy, retinopathy, neuropathy and microangiopathy are consequences of the metabolic disturbances seen in the disease and that the prevalence of these debilitating complications correlates with the duration and degree of hyperglycemia in both insulin-dependent diabetes mellitus (IDDM) and non-IDDM. In 1974, Lee et al. (4) reported that kidneys transplanted into diabetic rats developed vascular complications of diabetes and kidneys from diabetic donors transplanted

into nondiabetic recipients showed no further progression and, in some, regression of the lesions. Rasch (5-7) demonstrated renal mesangial and basement membrane thickening and an increase in albumin excretion in poorly controlled chemically induced diabetic rats and reported the absence of such lesions in rats receiving intensive insulin therapy. In a classic study, Engerman et al. (8) showed that dogs well controlled with two daily injections of insulin for 60 months developed fewer retinal lesions than dogs intentionally poorly controlled with a more conventional insulin regimen. Several investigators have reviewed animal and prospective human studies which support or deny the relationship between metabolic control of the disease and the course of diabetic microangiopathic or neurologic complications (9-12). The consensus was that the better the blood glucose control, the slower the rate of progression and severity of the lesions. This conclusion justifies the search for better methods for achieving excellent control of blood glucose levels.

At present, management of IDDM involves a therapeutic balance of caloric intake, energy expenditure and exogenous insulin which will avoid the extremes of hypoglycemia and hyperglycemia. Daily variations in diet, exercise, insulin absorption or resistance, and stress make good control of IDDM difficult to achieve with conventional insulin therapy. Intensive insulin therapy via multiple subcutaneous injections or insulin pump systems can prevent the wide swings in blood glucose levels, however, this requires highly-motivated individuals and most diabetic patients cannot or will not comply with this type of therapy (13,14). In addition, infection or skin irritation, chronic peripheral hyperinsulinemia, hypoglycemia and ketoacidosis are

associated risks (13,14). Thus, research efforts are directed towards "total endocrine replacement therapy" (Dr. D. Sutherland, Minnesota) via immediately vascularized pancreatic grafts or isolated islets of Langerhans. The implantation of functioning β-cells would permit precise homeostatic control of carbohydrate, fat and amino acid metabolism under all conditions of diet, exercise and stress. It is the most physiological approach to obtaining meticulous glycemic control.

PANCREAS TRANSPLANTATION

Initial attempts at transplanting the pancreas were unsuccessful. Vascular thrombosis, exocrine secretions and immunosuppressive corticosteroids had an unfavorable influence on the function of pancreatic allografts (15). With the introduction of cyclosporin-A (CyA) and new surgical techniques came a dramatic increase in the numbers of pancreas transplants performed. Since 1966, almost a third of those done worldwide were performed in 1984 (16). These were either whole organ or segmental grafts with attempts to control unwanted exocrine secretions by acute ductal ligation, intraductal injection of glues and polymers, leaving the duct open or establishing recipient gastrointestinal or genitourinary drainage (15). The variety of techniques used emphasize the difficulty in managing the exocrine secretions so as to avoid autodigestion of the gland and damage to surrounding tissues, as well as preventing complications such as vascular thrombosis. Each procedure has its own advantages and risks and a consensus as to which method is most appropriate has not been reached. The requirement for immunosuppression limits this procedure

as only those patients with diabetes-related complications that are or will be more serious than the side effects of long-term immunosuppression are candidates for pancreas transplantation. Other disadvantages include the very limited storage capacity of the pancreas and the inability to potentially use xenogeneic tissue. Currently, the estimated one year graft function rate is around 40% (17) and although the success rate is steadily improving, large-scale clinical application is still limited by technical difficulties and a requirement for continuous immunosuppression.

PANCREATIC ISLET TRANSPLANTATION

As many of the difficulties associated with pancreas transplantation are technical and related to the secretion of digestive enzymes by the exocrine pancreas, an alternative approach is to transplant only the endocrine component, free from contaminating acinar tissue. The islets of Langerhans are multicellular organs of specific cell types that work together as an integrated unit to provide precise control of glucose homeostasis. Although it is difficult to isolate from a single donor pancreas, a sufficient number of islets to effect a "cure", this approach to "total endocrine replacement therapy" has both practical and economic benefits over pancreatic transplantation. The small mass of the graft would make it easy to implant rapidly, safely and economically without high risk to the recipient if complications arise. Islet transplantation would also eliminate complications arising from vascular anastomoses and graft pancreatitis. In addition, the possibility exists for the establishment of tissue banks, the use of immunologically privileged sites and xenogeneic tissue. The

reduction of graft immunogenicity by manipulation of islets *in vitro* and thus, transplantation without continuous recipient immunosuppression is likely. This would greatly extend the eligible patient population beyond those with severe vascular and renal complications.

In 1965, Moskalewski (18) used collagenase, an enzyme complex derived from *Clostridium histolyticum*, to isolate islets from a guinea pig pancreas. In 1967, Lacy and Kostianovsky (19) increased the yield of islets by mechanically disrupting rat pancreas with a ductal injection of a salt solution prior to collagenase digestion. They also used a discontinuous sucrose density gradient to separate islets from acinar debris and noted some cellular damage in *in vitro* metabolic studies. Lindall *et al*. (20) substituted sucrose with Ficoll, a sucrose polymer of high molecular weight which provided a better osmotic environment for the islets. In 1972, Ballinger and Lacy (21), using the collagenase digestion and Ficoll purification technique, were first to obtain partial amelioration of the diabetic state by transplanting 400-600 isologous islets to the peritoneal cavity or thigh muscle of diabetic rats. Reckard *et al*. (22) normalized the diabetic state by repeated injections of up to 600 islets to the peritoneal cavity. A major advance in islet transplantation came in 1973 when Kemp *et al*. (23,24) demonstrated that islets which only partially ameliorated diabetes when transplanted intraperitoneally could completely normalize diabetic rats when they were transplanted to the liver via the portal vein. Islet isolation by collagenase

digestion and Ficoll purification followed by intraportal implantation became the standard technique in rat islet transplantation.

ANIMAL MODELS

Diabetes-like syndromes, which simulate various aspects of the human disease, can be induced in animals by a number of methods such as pancreatectomy, chemical agents, or viral infection (25). Diabetes also occurs spontaneously in various species of animals. Most islet transplantation studies have been performed in rodents with diabetes experimentally induced by chemical agents, most commonly streptozotocin (SZ), a specific beta cell toxin.

Streptozotocin is the agent of choice for inducing diabetes in rodent models. It was originally produced by Streptomyces achromogenes and has antitumoral, antibacterial, and oncogenic as well as diabetogenic properties (25,26). SZ induces specific irreversible damage to β-cells within pancreatic islets presumably by decreasing levels of cellular NAD (nicotinamide adenine dinucleotide) via its nitrosourea moiety (26,27). When administered in a conventional single diabetogenic dose, islet β-cell destruction is essentially complete and a lasting hyperglycemia and glycosuria is observed within 24 hours (25).

In 1976, Like and Rossini (28) reported that SZ administered to mice in multiple subdiabetogenic doses induced extensive infiltration of inflammatory cells into the islets which resulted in a delayed but progressive loss of β-cells and, thus, hyperglycemia. The lymphocytic infiltration was similar to that observed in IDDM (28,29). Susceptibility to multiple low dose SZ-induced insulitis is dependent

on age (30), sex (31), species (29) and strain (31,32), with young male mice of certain strains most susceptible. Islet transplantation studies in this model have shown that transplantation of syngeneic islets to the spleen (33-36) or kidney capsule (36,37) permanently reversed the diabetic state, although we and others (34,35) have noted spontaneous remissions of the diabetic state.

These reports and others (38,39) generated optimism that islet transplantation could reverse diabetes with an immune pathogenesis. Other investigators, however, have reported that islets, transplanted into animals with spontaneous diabetes, are destroyed by a recurrence of the original autoimmune disease (40,41).

TRANSPLANTATION SITES

The diabetic state has been reversed in inbred strains of rats following islet transplantation into the peritoneal cavity (21,22), liver via the portal vein (23,24), spleen (42,43), muscle (44), renal subcapsular space of the kidney (45,46), testes (47), brain (48,49) or a peritoneal-omental pouch (50). Intratesticular (47,51), intracerebral (48,49) and renal subcapsular sites (46) have an immunological advantage. The liver has been a preferred site in rodent models because it provides a rich and immediately available blood supply that nourishes the cells until engraftment occurs and because the regulatory hormones secreted by the islets enter the portal venous circulation where they achieve their maximal metabolic effect (52). The spleen (42,43) and a peritoneal-omental pouch (50) also fulfill the criteria for drainage to the portal circulation and clinically may be less dangerous to the recipient.

The renal subcapsular space is a popular alternative to the portal vein route for normalizing the diabetic state in rodents even though it lacks portal drainage. Advantages, both experimentally and clinically, are that it is easily accessible for insertion and subsequent removal of the graft for histological purposes or if complications, such as infection, arise clinically. Experimentally, it is easy to ascertain that normalization of the diabetic state was due to the graft and not regeneration of host islet tissue. Vascularization of islets placed beneath the kidney capsule occurs within 48 hours (53) and if a renal portal shunt is performed after islet transplantation, intravenous glucose tolerance test K values can be increased closer to normal (52,54). A recent study by Woehrle et al. (55) has shown that the kidney capsule is also beneficial in preventing both islet rejection and recurrence of the autoimmune disease in spontaneously diabetic BB rats.

Studies on islet transplantation site have indicated that a minimum islet mass is necessary to successfully normalize diabetes in any recipient, and transplanting in numbers greater than this will provide a functional reserve to compensate for any loss. Successful islet transplantation will also depend on the degree of tissue engraftment achieved. This will depend on the site of transplantation and the degree of tissue dispersion at this site as the islets must survive by nutrient diffusion until vascularization can occur.

EFFECT OF ISLET TRANSPLANTATION ON THE COMPLICATIONS OF DIABETES

The initial reports on islet transplantation indicate that provided adequate numbers of isologous islets are transplanted, the

metabolic defects (hyperglycemia, glycosuria, polyuria, weight loss) of experimental diabetes mellitus can be reversed. The rationale for islet transplantation is, however, the prevention and/or reversal of the chronic complications associated with this metabolic disorder. Several investigators have shown that islet transplantation can reverse the glomerular lesions (56,57), retinal changes (57,58) and autonomic neuropathy (59) that are observed after prolonged diabetes. Gray and Watkins (60) have prevented cataract formation and a variety of the renal and retinal abnormalities that occurred in diabetic controls by islet transplantation. Hoffman et al. (61) demonstrated that, in contrast to good control achieved with exogenous insulin, transplantation of fetal pancreas prevented glomerular basement membrane thickening in SZ-induced diabetes. In summary, islet isografts can normalize the clinical indicators of diabetes and are capable of preventing or reversing early microvascular complications that develop secondary to experimental diabetes.

VULNERABILITY OF ISLETS TO REJECTION

Early studies showing that endocrine tissue such as parathyroid, ovary and thyroid were only weakly immunogenic generated optimism for the success of islet allografts (62). Unfortunately, isolated islets do not enjoy the immunologically privileged status of other endocrine tissue (63). They are, in fact, extremely vulnerable to rejection effector mechanisms (22,64-67). Transplantation of histoincompatible islets in nonimmunosuppressed rats resulted in median survival times of 3-4 days whereas skin, kidney and heart allografts transplanted across the same major histocompatibility complex (MHC) barriers survived 8-12

days (22,66,68). Morris et al. (67) reported that intraportal transplantation across major barriers resulted in rapid rejection and was very difficult to suppress with a variety of immunosuppressive regimens. It also appears that isolated islet allografts are at an immunological disadvantage when compared to vascularized pancreas allografts (68-70). Perloff et al. (70) found that isolated islets were rejected 3-5 days after transplantation into either MHC-compatible or incompatible diabetic rats while vascularized pancreas survived 7.6 days in MHC-incompatible hosts and a median of 16.5 days in MHC-compatible hosts.

Both humoral and cellular mechanisms are responsible for islet allograft reje[illegible] Islet grafts, established in immunosuppressed or tolerant reci[illegible] are rapidly rejected following the injection of donor-specific [illegible]lo-antiserum or lymphoid cells (63-65). Several reasons have been suggested for the extremely rapid rejection of isolated islet grafts. These include:

1) islets may have an immunogenic tissue specific antigen or, collagenase digestion during the isolation procedure exposes antigens not normally expressed (69)

2) only marginally effective numbers of islets are transplanted so that loss of only a few will result in hyperglycemia and appear as graft destruction (71)

3) transplanting a dispersed cell preparation increases susceptibility to humoral factors over that of vascularized organ allografts (63)

4) islet preparations are contaminated with highly immunogenic exocrine debris (72)

Remarkable progress in pancreatic islet transplantation has been made in the last decade. Islet transplants have reversed diabetes in rodents and larger animal models and have prevented or reversed early diabetic complications in recipients. There still remains, however, three barriers to the large-scale clinical application of islet transplantation:

1. The ability to repeatedly isolate a sufficient islet cell mass from a single donor
2. Islet preservation prior to transplant
3. Prevention of immune rejection post-transplant

The isolation procedure must yield a critical mass of islets without affecting the viability of the tissue. The tissue must be stored until the recipient is ready for transplantation and then successfully placed in a site where engraftment is rapid and where the hormones secreted will exert their maximal metabolic effect. Finally, graft rejection must be prevented without continuous recipient immunosuppression.

PANCREATIC ISLET ISOLATION

A major obstacle to clinical application of pancreatic islet transplantation is the technological inability to repeatedly isolate and purify sufficient quantities of human islet tissue. Since the development of the standard islet isolation technique of collagenase-digestion (18,19) and Ficoll purification (20,73), few investigators have developed techniques which have greatly increased yields from the rodent pancreas. Some have reported higher yields using donor pretreatment with pilocarpine (74), DL-ethionine (75) or seleno-dL-methionine (76) to reduce exocrine enzyme content. Others

have used ductal distension with collagenase (77), centrifugation on Percoll (78) or bovine serum albumin (79) gradients, or pancreatic perfusion with neutral red to exclude lymphatic and acinar contamination (80). Still, based on the early estimates of the number of islets per rat pancreas, yields of only 5-10% are regularly obtained (15).

Islet tissue sources other than the adult pancreas have been investigated. Fetal and neonatal tissue are good sources because they consist of a high ratio of endocrine to exocrine tissue and have the ability to differentiate into endocrine cells (81,82). Rodent fetal pancreas has the potential to differentiate in organ-culture (81,82) or after transplantation in a normoglycemic host (83). If fetal pancreas is transplanted to a diabetic host, insulin is needed to stimulate growth of the endocrine cells (84). Unfortunately, multiple donors are required and there is usually a lag phase of several months before amelioration of the diabetic state. In addition, fetal pancreas is more immunogenic than isolated islets (85). Overall, these immature tissues do not offer more advantages than adult tissue and may not be applicable to large-scale human islet transplantation.

Rodent islet isolation procedures had to be modified to accommodate the more compact and fibrous canine and human pancreases (86). Most preparations from large mammalian pancreases consist of islets entrapped within exocrine tissue (87). Transplantation of these dispersed canine pancreatic microfragments has been successful in ameliorating diabetes on a one to one basis (88-90). Recently, major advances in achieving high yields of purified canine and human islets have been made (91,92). Because purified islet preparations would

increase the ability to alter the immunogenicity of the tissue and reduce the implant volume, these techniques will greatly facilitate clinical trials of pancreatic islet transplantation.

ISLET PRESERVATION

Temporary holding methods for pancreatic islet tissue would allow time for tissue typing, crossmatching and selecting or pre-treating an appropriate recipient. Islets of Langerhans have been successfully preserved by simple cold storage, tissue culture and cryopreservation (93). Tissue culture or cold storage methods for islet preservation are feasible for short periods, however, (i) there is an associated progressive loss of viability, (ii) time-consuming, meticulous media changes are necessary, (iii) contamination is an ever-present risk, and (iv) large volumes of tissue are difficult to handle especially for transportation between centers. These inherent problems could be eliminated by cryopreservation. Successful short or long-term storage by cryopreservation would greatly facilitate steps toward the clinical stage of islet transplantation.

Establishing tissue banks of cryopreserved islets would remove time restraints on the selection and preparation of a suitable recipient and on the transportation of tissue from one transplant center to another. Unlike tissue culture and cold storage, there is not a progressive loss of tissue viability with extended storage periods and the contamination risk is much less. There would be ample time for an <u>in vitro</u> assessment of viability, islet tissue could be accumulated from several donors if necessary, and xenogeneic tissue, if feasible, could be readily available. Like tissue culture (94),

cryopreservation can aid in the purification of islet tissue by selectively destroying exocrine tissue (95-97). In an attempt to minimize islet rejection, the diabetic recipient of a kidney allograft could be given frozen-thawed islets from the same donor once a tolerant state is established. There have also been suggestions in the literature that cryopreservation could minimize islet immunogenicity (98,99). These potential benefits for the clinical application of islet transplantation have led to numerous reports on the cryopreservation of rodent, porcine, canine or human islet tissue and, primarily in rodent models, on the ability of frozen-thawed islets to reverse the course of experimentally-induced diabetes.

Successful cryopreservation of cells or tissues depends on several inter-related critical factors. These are (1) the extent to which the cryoprotective additive has permeated the cell prior to freezing, (2) cooling rate, (3) warming rate, and (4) the osmotic problems encountered when the additive is removed after thawing (100,101). According to Mazur's two-factor hypothesis (102), cells cooled too rapidly will be damaged by intracellular ice crystals which grow upon warming. Slow cooling exposes the cell to damaging "solution effects" such as high solute concentrations, pH changes, or osmotic shrinkage beyond a critical minimum volume. Additives such as DMSO and glycerol protect against these kinds of slow cooling injuries provided they are not used in toxic concentrations or do not induce osmotic damage when the cells are returned to isotonic media after thawing (100-102). These critical cryobiological factors as well as other factors specific to islets (method of isolation, pre-freeze or post-thaw culture

periods, and viability tests) affect cell survival and make comparisons of freezing protocols difficult.

In 1976, Mazur et al. (103) preserved 17-day old fetal rat pancreases by incubating in 2 M DMSO, cooling at 0.3°C/min to -196°C, slowly warming, and diluting with isotonic media in the presence of 0.75 M sucrose, a nonpermeating solute which limits osmotic swelling as the DMSO leaves the cell. They reported that cryopreserved pancreases synthesized 80% as much protein as unfrozen controls and became vascularized after transplantation into non-diabetic adult recipients. Fetal rat pancreas, cryopreserved by this technique and implanted beneath the kidney capsule, reversed diabetes in adult recipients (104). In 1977, Rajotte et al. (105) cryopreserved isolated rat islets with a technique similar to that used for fetal rat pancreas. They demonstrated that islets, slowly cooled at 0.5-0.7°C/min with 2 M DMSO and rapidly thawed, could secrete insulin in response to high glucose and improve the diabetic state of one rat following intraportal transplantation. A series of papers by Rajotte and colleagues (95,105-111) have indicated that, for rat, canine or human islets of Langerhans, a slow cooling rate is necessary for maximum recovery both in vitro and in vivo.

In 1981, Rajotte et al. (106) demonstrated the feasibility of low-temperature banking of islets by freezing islets at one center (Edmonton, Canada) and transporting them to St. Louis, Missouri where they were thawed and transplanted to the liver via the portal vein of SZ-induced diabetic rats. In this study, islets equilibrated in 2 M DMSO, cooled at 0.25°C/min to -75°C and thawed at 7.5°C/min were able to reverse the diabetic state but rats receiving islets cooled at

1°C/min remained diabetic. When controlled cooling was interrupted at -40°C rather than -75°C, and islets were thawed at 200°C/min (95), a better *in vivo* response was obtained because the islets were not exposed to "solution effects" injury for a prolonged period. Three thousand islets slowly cooled to -40°C (as compared to 5000 islets cooled to -75°C) gave the same clinical responses as 3000 fresh islets after transplantation into diabetic rats. Rats receiving cryopreserved islets, however, still had abnormal glucose tolerance tests (95,106,107).

Other investigators have also successfully cryopreserved rodent islets with slow cooling rates (112-115). McKay and Karow (112) have demonstrated that rat islets cooled to -75°C at 0.3°C/min but not 3, 14 or 48°C/min release insulin in a biphasic pattern in response to glucose challenge. Cooling rates less than 1°C/min are in agreement with the theoretical calculations of Mazur and the optimum cooling rates for mammalian embryos (101) and fetal rat pancreas (103,116). Bretzel *et al.* (114,115) have cryopreserved rat islets by slow cooling in 10% (1.3 M) DMSO at 2°C/min to -35°C, then 6-7°C/min to -100°C in combination with rapid thawing. They reported glucose-stimulated insulin release similar to fresh islets and normalization of the diabetic state following syngeneic intraportal transplantation.

In contrast, Bank [illegible]), in 1979, determined that a rapid cooling rate (75°C/mi[illegible] [illegible]ptimum for isolated rat islets. Net insulin release on g[illegible] challenge after cooling in 1 M DMSO and slowly thawing at 3.5°C/[illegible] was reported to be 75% of the amount released by fresh islets. Mazur (100-102) predicts that this protocol would promote intracellular ice formation during rapid cooling and

recrystallization during slow thawing. Bank and Reichard (118) later reported greater success with a two-step cooling procedure in which cells are cooled rapidly to an intermediate sub-zero temperature and held for a specified time to allow osmotic shrinkage before rapidly cooling to -196°C. Andersson and Sandler have shown better survival when mouse islets were continuously cooled to -70°C at 5°C/min rather than 0.5°C/min (119-121). Recently, Sandler et al. (122) have compared cultured islet survival following cooling at 5, 15, or 25°C/min. In batch-type incubation, each group of cryopreserved islets released insulin in response to high glucose concentrations. Islets cooled at 25°C/min responded similar to controls in perifusion and proinsulin biosynthesis although insulin content was reduced by 30%. Intrasplenic transplantation of islets frozen at 25°C/min resulted in complete or partial normalization of diabetes in 7 of 9 mice.

Taylor and Benton (123) have recently frozen fully or partially DMSO-equilibrated islets with cooling rates of 0.3, 3.0, 10, 30, 60, 150 or >1000°C/min in conjunction with either slow or rapid thawing. Viability was assessed by insulin secretion during static incubation. However, functional survival was highly dependent upon cooling and thawing rates when islets were only partially equilibrated. Peak survival of fully equilibrated islets, at a cooling rate of 0.3°C/min with rapid thawing, was not much greater than recovery at 60 or >1000°C/min. They concluded that cooling rates are not major determinants of survival if the islets are fully equilibrated with 2 M DMSO prior to cooling. Further studies are necessary to confirm this hypothesis.

Direct comparisons of islet viability following the various freezing protocols reported for rodent islets of Langerhans are impossible because of many different, yet, inter-related variables. In addition to cooling and warming rates, the concentration or extent of permeation of the cryoprotectant, the immediate post-thaw handling of the tissue, the addition of pre- and post-thaw culture and the assessment of viability all influence the reported survival. Perifusion is the most sensitive in vitro method of assessing function (119) but the ultimate test of viability is the reversal of diabetes following transplantation. Cryopreservation of canine or human pancreatic fragments has also been reported and protocols still vary with cooling rates ranging from 0.3 (97,107-111) to 60°C/min (124). Rajotte et al. (95) found that application to these large models required some modification of the pre-freeze handling of the tissue. Clearly, cryopreservation of islet tissue is feasible and would eliminate the contamination risks and the time restraints associated with tissue culture techniques.

PREVENTING REJECTION OF TRANSPLANTED ISLETS

ALLOGRAFT REACTIVITY

Class I antigens of the major histocompatibility complex (MHC) (coded for by murine H-2 K/D or its human homologue HLA-A,B,C gene loci) are expressed on the surface of all nucleated cells. These antigens are targets in transplantation reactions and preferentially stimulate cytotoxic T-lymphocytes (125). Class II MHC antigens, products of murine I-r genes or similarly human D/DR genes, are expressed on cells of lymphoreticular origin such as T or

B-lymphocytes, macrophages or dendritic cells (125). These immune-response associated (Ia) antigens, the 'transplantation antigens' of graft rejection, play prominent roles in the immune response, activating lymphocytes of the helper T-cell subset and providing strong stimulation in mixed leukocyte culture (MLC) and graft versus host reactions (125,126).

The following concepts of allograft reactivity have been reviewed by Lafferty et al: (127,128).

(A) The Classical Concept

The classical view of allograft rejection describes transplanted tissue as the source of antigen which activates a cell-mediated (T-cell) response and triggers the rejection process in the graft. Transplantation antigen, whether Class I or Class II MHC antigen, was thought to be the major barrier to successful tissue transplantation. It followed that antigen recognition alone was sufficient for lymphocyte activation. Experimental observations, however, did not support this assumption. The isolation and characterization of the MHC antigens revealed that they are extremely weak immunogens and the mixed leukocyte reaction, an in vitro analogue of allograft rejection, showed that the capacity of allogeneic cells to stimulate T-cell activation was a function of metabolically active cells. This meant that, although they could still express antigen, ultraviolet (UVB) irradiated cells could not stimulate a response. These observations indicated that the triggering of the allograft response required more than the presence of foreign antigen.

(B) The Stimulator Cell Model of Immune Induction

In 1975, Lafferty and Cunningham (129) proposed a two-signal model for the triggering of an antigen-specific lymphocyte response. The first signal for T-cell activation is provided by antigen binding to the T-cell receptor and the second signal, an inductive molecule or cytokine, is provided by a metabolically active antigen-presenting cell (APC). Activated T-cells then undergo clonal expansion and proliferation. Thus, T-cells are activated when they interact with antigen on the surface of metabolically active APC because both signals (antigen binding and the release of cytokine) are provided.

Antigen-presenting cells are of lymphoreticular origin and tend to possess Ia-antigens. T-lymphocytes, macrophages, and dendritic cells (130), which are known to be potent stimulators of the MLC, are active antigen-presenters. According to this stimulator cell model, viable alloantigen-bearing cells that can not provide the second signal, such as fibroblasts, erythrocytes, or platelets, will not be immunogenic (127,128).

ANTIGENICITY OF ISOLATED ISLETS

The expression of Class I antigens and the lack of Class II (Ia) antigens on the β-cells of mouse (131-133) as well as rat (133-136) and human islets (133,136,137) has been reported. Rabinovitch et al. (135) detected Ia antigens extensively on lymphocytes, macrophages, and capillary endothelial cells within freshly isolated rat islets. This group (136) and Hart et al. (134) later reported that rat and canine islet endothelium is Ia-negative, however, human islet endothelial cells extensively express Ia antigens (136,137). Gores et al. (138)

detected a mean of 14.9 (range 5-24) Ia^+ cells within untreated mouse islets. Shienvold et al. (136) found 0-5 Ia^+ cells per rat or canine islet. Pipeleers et al. (139) has recently purified Ia-positive cells by fluorescence-activated cell sorting and reported that this population constitutes approximately 1% of all islet cells. These immunogenic lymphoid cells have been identified as macrophages by some investigators (136). Others (134) have concluded that the densely staining Ia^+ cells found within isolated rat islets were likely interstitial dendritic cells.

Regardless of the specific cell type involved, isolated islets are extremely vulnerable to rejection effector mechanisms. A means of preventing rejection must be established, however, unlike the transplantation of heart, liver or lung in which the organ is required for life, the advantages of islet transplantation as an alternative to exogenous insulin therapy must be carefully weighed against the potential side effects of immunosuppression. For the majority of diabetics whose complications are not yet life-endangering, immunosuppression poses a greater risk. Therefore, in order for pancreatic islet transplantation to have large-scale clinical application, the requirement for recipient immunosuppression must be substantially reduced or eliminated.

The many attempts made to prevent the rejection of transplanted islets involve either treatment of the recipient or treatment of the graft prior to transplantation. These include:

1) Minimizing histoincompatibility

2) Generalized immunosuppression

3) Induction of tolerance

4) Total lymphoid irradiation

5) Immunologically privileged sites

6) Immuno-mechanical barriers

7) Reduction of graft immunogenicity

1) Minimizing histoincompatibility

Minimizing the histocompatibility differences between donor and recipient, an approach based on the classical concept of allograft reactivity, has not been as effective for islet allografts as it has been in experimental and clinical organ transplantation. Initial attempts at allografting islet tissue revealed the extremely rapid rejection of even weakly histoincompatible islets (140). H-2 compatible islets, in fact, survived only a few days longer than strongly histoincompatible tissue (140) and when donor and recipients differed only with respect to the weak H-Y antigen, islets were still rapidly rejected (141). In certain mouse strains, female mice can reject isologous male islets more rapidly than skin grafts (less than 7 days as compared to 20-29 days).

Some murine islet allografts disparate at the entire H-2 complex are accepted long-term (142,143). Selawry (144) found that allograft acceptance of purified rat islets was also independent of the degree of histoincompatibility as determined by the magnitude of mixed lymphocyte culture responses in vitro. Morrow et al. (143) genetically removed class II differences using recombinant strains of mice and found that mice disparate for class I antigens alone rejected islet allografts. This study implies that Ia antigen recognition is not an absolute requirement for T-cell activation. Steffes et al. (145) transplanted

islets between mice which were identical at K and D regions but differed in I and S regions and reported an unpredictable response as some were accepted and others were rejected. Zhu et al. (146) reported that the pattern of allograft rejection in congenic mouse strains was dependent on the specific donor and recipient genotypes, regardless of the absolute H-2 antigenic disparities involved. Thus, results are greatly influenced by the strains chosen.

2)Generalized chemical immunosuppression

A number of pharmacological agents, alone or in combination with others, have been able to successfully prevent the rejection of rodent heart or renal allografts but have failed to significantly prolong islet allograft survival across major or minor barriers (67,147,148). The search for new immunosuppressive agents, however, has not been abandoned (149,. Of those immunosuppressants in current use, recipient-species-specific anti-lymphocyte serum (ALS) and cyclosporin A have been the most effective in rodent islet allografts.

(A) Anti-lymphocyte serum

ALS has prevented rejection of rodent allografts with considerably greater success than immunosuppressive drugs. In 1973, Reckard et al. (22) found that a five day treatment of ALS prolonged survival of rat allografts from 8.5 to 30.5 days. Daily injections of this agent prevented rejection of rat islet allografts for greater than 200 days (150). Many investigators have since been able to significantly prolong rat islet allograft survival across minor or major histocompatibility barriers with short-term treatment of ALS (67,147, 151,152).

ALS has also been effective in prolonging survival of concordant (rat to mouse) islet xenografts. An initial report showed xenograft survival extended to 24 days (65) following intraperitoneal islet transplantation and repeated injections of rabbit anti-mouse thymocyte serum. A single injection of ALS at the time of transplantation extended survival of intraportal xenografts to 49 days (153) compared to only 9.7 days for intrasplenic xenografts (154). Many groups have obtained prolonged or indefinite allo- or xenograft survival using ALS in combination with other immunomodulating regimens.

(B) Cyclosporin A

Cyclosporin A is a cyclic polypeptide composed of 11 hydrophobic amino acids (155-157). This fungal metabolite has potent immunosuppressive properties and has been used successfully in experimental and clinical organ transplantation (155-157). The mechanisms of CyA action are still under study. Generally, CyA exerts its effect in the early phase of T-cell activation, inhibiting the activation of lymphocytes by blocking interleukin-2 production (158). More specifically, CyA may act at the level of interleukin-2 gene transcription (159) or block transmission of the antigen signal that activates lymphocyte-encoding gene expression (160).

Cyclosporin renewed interest in pancreas transplantation as it reduced or eliminated the need for diabetogenic corticosteroid immunosuppressants (157). It has not, however, proven to be as successful in preventing rejection of pancreas allografts as it is with other organ grafts (156,157), and pancreas allografts in the presence of CyA survive considerably longer than comparable islet allografts (157,161,162). An initial study by Vialettes et al. (163) showed that

CyA, administered orally for 2 weeks at a dose of 50 mg/kg/day, extended islet allograft survival across a major histocompatibility barrier to 23.4 days. Other investigators have reported toxic effects at less than this dose (67,161,162,164) and, in contrast to studies which show indefinite renal allograft acceptance following a short peri-transplant period of CyA (165), islet allografts were rejected after the drug was withdrawn (67,149). In contrast to these previous results, Dibelius et al. (80) prolonged rat islet allograft survival across a major histocompatibility barrier with an injection of CyA (30 mg/kg) given at 0, 1 and 2 days after transplantation. Graft survival, assessed by serum glucose levels, glucose tolerance tests and histology, was significantly prolonged to 28-150 days. In canine pancreatic fragment allotransplantation, CyA was shown to be more effective and less toxic than azathioprine/steroid immunosuppression in preventing rejection (166).

The failure of rodent islet allografts in the presence of CyA may, in part, be related to its deleterious effect on the function of islet tissue (167-170). Morphological and functional changes have been noted in pancreatic β-cells of Wistar rats treated with CyA (50 mg/kg) for 7 days (168) or 3 weeks (170). Both groups reported decreases in islet insulin content to 70% as well as impaired glucose tolerance and hyperglycemia.

In summary, generalized immunosuppressive protocols can significantly prolong islet allograft survival in certain donor-recipient strain combinations, however, they are not as effective as for other types of organ allografts unless used in such high doses

that toxicity becomes a problem. Specific immunosuppressive therapy would be more feasible for clinical islet transplantation.

3)Induction of tolerance

In rodents, the induction of a donor-specific unresponsive state will result in indefinite survival of allogeneic tissue. Classic induction of tolerance by inoculation of alloantigens in neonates is donor-strain specific and has been shown to induce permanent acceptance of islet allografts in some studies (64) but not others (70). Active induction of tolerance in adult animals by inoculation of cell extracts of donor genotype prior to transplantation is more difficult. Faustman et al. (171) has been able to show permanent acceptance of H-2^k islet allografts in 80% of H-2^b mice which received, 2-3 weeks earlier, donor red blood cells treated with anti-Ia^k antiserum to remove contaminating Ia^+ leukocytes. All allograft recipients given untreated donor red blood cells prior to islet transplantation rejected their grafts within two weeks. Administration of UVB-irradiated donor spleen cells following the transplantation of cultured islets induced specific immunological tolerance in that it prevented the induction of rejection by subsequent administration of viable donor spleen cells (85,172). A spontaneous form of tolerance in several murine recipients of cultured islet allografts has also been noted (173). These animals were resistant to challenge with donor strain peritoneal cells after a prolonged period of graft function. Thus islet allograft tolerance can be induced in adult rodents without recipient immunosuppression. Studies into the mechanism of the induction of immunological

unresponsiveness may aid in the application of these approaches in larger animal models.

The reports showing that short-term cyclosporin A treatment (161) or discontinuation of CyA (162) resulted in rejection of transplanted islets indicate that islets of Langerhans are not capable of inducing tolerant states as easily as other organs. Mice bearing long established anti-Ia-treated islet allografts rejected donor-specific skin grafts and did so even more rapidly than controls (174,175). The rejection of islet allografts followed shortly after. Recipients of established cultured islet allografts, however, have been shown to accept uncultured islets (176). One way of preventing the rejection of transplanted islets has been to transplant into animals bearing an established renal allograft, making use of the unresponsive state it has generated. Reece-Smith et al. (177) established renal allografts across a major histocompatibility barrier with a two week treatment of cyclosporin and then, 100 days later, permanently reversed SZ-induced diabetes by transplanting donor-strain islets beneath the capsule of the donor-strain kidney. No further immunosuppression was necessary. Islets, including third-party strain islets, were rapidly rejected if they were transplanted at the same time as the kidney allograft indicating that the tolerant state was required for islet graft survival. Permanent donor-strain allograft survival was also achieved in 50% of recipients with established spleen allografts and in 100% of recipients if CyA was given for 7 days at the time of islet transplantation (178). Procedures like these would likely allow successful transplantation of cryopreserved islets into a patient who has previously accepted a renal allograft from the same donor; however,

like pancreas transplantation, they would be available only to diabetic patients with end-stage complications.

4)Total Lymphoid Irradiation

Fractionated total lymphoid irradiation (TLI) is the localized, cumulative, high-dosage irradiation of lymphoid tissue followed by inoculation of allogeneic bone marrow. It is one of the most potent forms of recipient immunosuppression, inducing specific unresponsiveness to allogeneic tissue. Using this procedure, Mullen and co-workers achieved permanent graft survival of fetal rat pancreas transplanted beneath the renal capsule across both minor (179) and major histocompatibility barriers (180). They found that low dosages of both TLI and donor bone marrow were sufficient to achieve indefinite survival across minor barriers and higher dosages of each were necessary to achieve this effect across major histocompatibility barriers. Also across a major barrier, Britt et al. (181) established permanent and specific immunological unresponsiveness to isolated rat islets in 2 of 3 rats that survived a protocol of high-dose TLI and donor-strain bone marrow infusion. In a hamster-to-rat xenograft model, Nakajima et al. (182) demonstrated an increase in islet graft survival with increasing total doses of TLI; extending survival to 30.0 days with a total dose of 1200 rad. Thus, allogeneic adult islets or fetal pancreas can survive indefinitely across major histocompatibility barriers using a combination of high dose TLI and donor bone marrow infusion. No further immunosuppressive treatment is necessary, however, high mortality was often observed.

5) Immunologically privileged sites

It has long been known that there are certain "immunologically privileged" sites in the body that will vascularize and sustain small tissue allografts for long periods of time. The host is immunologically unaware of their presence. Such sites include the anterior chamber of the eye, the testis and the brain. There has been some suggestion that the renal subcapsular space has an immunological advantage over the liver. Reece-Smith et al. (46,183) found that allogeneic rat islets implanted beneath the kidney capsule survived twice as long as those transplanted intraportally and that the survival of subcapsular islets was prolonged more easily with CyA than intraportal islets. Survival of rat to mouse islet xenografts beneath the renal capsule was also extended twofold over intraportally placed xenografts (184,185). That the renal subcapsular space is an immunoprivileged site relative to the liver has been disputed by Gores et al. (186) using a congenic murine islet allograft model. These claims may depend on the histocompatibility barrier between the donor and recipient strains used.

Intratesticular islet allo- and xenografts (47,51) have shown prolonged survival compared to grafts placed intraportally or beneath the kidney capsule. In an allograft model, islets cultured for 4 days failed to reverse diabetes when transplanted to the liver or renal subcapsular space but survived for more than 50 days in intra-abdominally placed testis (47). Intratesticular xenograft survival was extended to a mean of 30.8 days, with 3 of 12 grafts lasting more than 60 days, while comparable xenografts transplanted

intraportally or beneath the renal capsule survived for 7 and 11.2 days respectively (51).

The brain also provides a suitable site for implantation of rat islet tissue. Tze and Tai (48,49) have shown indefinite survival of pancreatic endocrine cells after implantation intracerebrally or intrathecally to the subarachnoid space. These grafts were susceptible to immune rejection following challenge with donor strain skin grafts (49). The outcome of allograft survival, however, was dependent on the type of donor tissue transplanted as allotransplantation of whole islets to these sites were rejected in 7 of 9 (48) and 3 of 8 (49) cases.

The immunoprivileged status of the testis and brain has been demonstrated in rodent models however, these sites may not be applicable to human islet transplantation.

6) Immuno-mechanical barriers

Various techniques for enclosing islets within semi-permeable membranes so as to allow hormonal diffusion and protection against rejection have been developed and reviewed by Scharp et al. (187). Early microcapsular systems survived in vivo for no longer than 2-3 weeks because of poor biocompatibility of the materials used (187,188). Failures were due to inflammatory responses induced by polyethyleneimine (188). Using a semi-permeable alginate-polylysine membrane, O'Shea et al. (188) implanted 4500 encapsulated allogeneic islets into the peritoneal cavity of SZ-induced diabetic rats, reversing the diabetic state for up to one year. Unencapsulated islets were rejected in less than two weeks. Darquay and Reach (189) have

shown that alginate-polylysine membranes protect rat islets from cytotoxic anti-islet antibodies in vitro and O'Shea and Sun (190) reported greater than 80% xenograft survival at 50 days with a mean of 80 days. These immuno-isolation devices, by eliminating the cells or molecules that mediate rejection, show great promise for eliminating the need for recipient immunosuppression, however, applications are still severely limited by biotechnological problems.

7) Minimizing immunogenicity

In 1975, Lafferty et al. (191) reported that thyroid tissue became less immunogenic after a 12 day period of tissue culture in an atmosphere of 95% O_2 and 5% CO_2. Organ culture results in the degeneration of the vascular bed and blood elements within the cultured tissue (192). This degeneration or, alternatively, a loss of graft antigen could result in a reduction of tissue immunogenicity. Talmage et al. (193) found that the injection of donor-strain peritoneal exudate cells to mice bearing established cultured thyroid allografts resulted in rejection of the tissue. This indicated that the graft still carried recognizable antigen and that the reduction of thyroid immunogenicity likely resulted from the sensitivity of leukocytes to high oxygen tensions (194).

These studies led to the return of the passenger leukocyte concept, initially proposed by Snell (195) in 1957, which suggested that leukocytes residing in the donor tissue provide the major barrier to successful tissue transplantation. Now based on the stimulator cell model for allograft reactivity, this concept suggests that the removal of the passenger leukocytes (Ia^+-cell populations) from within the

graft tissue prior to transplantation will result in prolonged allograft survival (127). Many of the attempts made to prevent the rejection of islet allografts are based on this concept. These attempts have included pre-transplant culture in high oxygen atmospheres or at low temperatures, anti-Ia antisera and complement or ultraviolet irradiation..

(A) Pretreatment with culture

(i) Allograft studies

Initial attempts at culturing rodent islets in high oxygen atmospheres were unsuccessful as isolated islets were rapidly destroyed (45,152). This extreme sensitivity of islets to 95% O_2 was avoided if islets were aggregated into clusters of about 50 islets (45). Mouse islets isolated from cyclophosphamide-treated donors and cultured in clusters for 7 days at 37°C showed no evidence of rejection for up to 420 days after implantation beneath the kidney capsule of allogeneic nonimmunosuppressed mice (45,85). In contrast, uncultured islet allografts were heavily infiltrated with mononuclear cells 4 days post-transplant (45,85). Allotransplantation of these cultured islets resulted in the reversal of SZ-induced diabetes for greater than 100 days (85) and the removal of the graft-bearing kidney resulted in a rapid return to the diabetic state (196). Intrasplenic transplantation of allogeneic islets, kept in 37°C culture and an air-CO_2 atmosphere for 4 weeks, partially or in some cases completely reversed diabetes for several weeks in nonimmunosuppressed mice (197).

Based on a report that human lymphocytes cultured for 4 days at 22°C lost their ability to stimulate a mixed leukocyte reaction in

vitro (198), Lacy et al. (151,152,199) transplanted islets intraportally across major histocompatibility barriers in a rat allograft model. They found that rat islets cultured for 7 days at 24°C survived for periods greater than 100 days provided a single injection of rabbit anti-rat lymphocyte serum (RALS) was given at the time of transplantation. Tze and Tai (200) found that a 7-day pretransplant culture of rat islets at 26°C but not at 32°C or 37°C prolonged the survival of allografts across a major barrier in nonimmunosuppressed rats. In contrast, Rabinovitch et al. (135) transplanted rat islets, cultured for 7 days at 25°C or 37°C, to allogeneic recipients which received RALS at the time of transplantation. They found that islets cultured at 37°C survived significantly longer (>27.0 days) than those cultured at 25°C (10.0 days). Also in a rat allograft model, Tucker et al. (201) obtained 100% survival of intraportal islet allografts in nonimmunosuppressed rats by keeping islet clusters in 37°C culture and an atmosphere of 95% O_2:5% CO_2 for 7-10 days.

Recent studies have shown that across major barriers, where pretreatment with culture was inadequate to prevent allograft rejection, the combination of culture and short-term immunosuppression with CyA had a synergistic effect and prolonged survival. Lacy's group could not prevent allograft rejection by low-temperature culture alone but achieved 100% survival at 60 days when CyA (30 mg/kg) was injected subcutaneously at 0,1 and 2 days after transplantation (202). The CyA treatment alone resulted in 29% survival at 60 days. Simeonovic et al. (203) have shown that the combination of donor pretreatment with cyclophosphamide, islet culture in 95% O_2:5% CO_2 at 37°C for 7-10 days,

and short-term (12-day) recipient immunosuppression with CyA (50 mg/kg/day) was essential for 100% survival of islet allografts in outbred mice. This result was based on histological examination. Transplantation in outbred mice resulted in complete or temporary reversal of diabetes in 80% of recipients, however only 38% remained normoglycemic after the withdrawal of CyA.

(ii) Xenograft studies

Islet pretreatment in tissue culture has also been able to prolong the survival of concordant (rat to mouse) islet xenografts. Lacy et al. (153) transplanted 450 rat islets which had been cultured for one week at 24°C to the liver of diabetic mice. Cultured xenograft survival was prolonged to greater than 59.5 days (as compared to 49.0 days for fresh xenografts) when mice received a single injection of rabbit anti-mouse lymphocyte serum (MALS). If MALS was combined with RALS, survival was extended to greater than 120 days. Survival was shown to be site-dependent when similarly cultured islets were transplanted to the spleen of mice receiving an injection of MALS and RALS at the time of transplantation (154). Intrasplenic xenografts were rejected by 7 days. Lacy et al. (184) also prolonged islet xenograft survival by culturing rat islet clusters (megaislets) for 7 days in an atmosphere of 95% O_2: 5% CO_2 at 37°C. Megaislet xenografts containing 750-800 islets had a mean survival time greater than 52.1 days (36% survival at 70 days) following implantation beneath the kidney capsule of diabetic mice. Similarly cultured islets survived for a mean of 16.9 or 7.0 days when implanted into the liver and spleen, respectively. In this study, the injection of MALS and RALS at

the time of cultured megaislet transplantation did not prolong survival over that of cultured megaislets alone. This group later found that 7-day, 37°C culture alone prolonged rat islet survival beneath the kidney capsule of nonimmunosuppressed mice (185). The mean survival time, without high oxygen tensions or ALS, was greater than 46.1 days, with 30% survival at 60 days. Neonatal rat islets obtained by a 37°C culture-isolation technique reversed diabetes for 4-17 weeks under the kidney capsule of nonimmunosuppressed mice (204). Cyclosporin A was found to be ineffective in prolonging fresh islet xenograft survival in a dose of 25 mg/kg for up to 7 days post-transplant (205). Three-day peri-transplant administration of 50 mg/kg CyA moderately prolonged xenograft survival to 21 days but when this therapy was used in conjunction with islets cultured at 24°C for 7 days, survival was extended to greater than 56 days, with 23% functioning at 100 days (205). Low-temperature culture alone was not effective in prolonging xenograft survival.

(iii) Support for the concept

These attempts to prolong islet allo- and xenograft survival by tissue culture are based on the passenger leukocyte concept. Support for the hypothesis that the reduction of immunogenicity is due to the depletion of immunostimulatory passenger lymphoid cells within the islet tissue comes from studies in which established cultured islet allografts are rejected by administering lymphoid cells to the recipient. Lacy et al. (153) found that peritoneal exudate cells of donor-strain could induce the rapid rejection of established rat islet allografts. Third-party peritoneal exudate cells did not affect the

allograft. In another study, this group concluded that rejection was induced by the macrophage population of peritoneal exudate cells (206). Splenic T-cells but not B-cells also induced rejection (173) but did so by a different mechanism as the time necessary to reach pre-transplant diabetic levels was much longer and the response was not donor-strain dependent.

In a mouse allograft model, Bowen et al. (173) demonstrated the vulnerability of established cultured grafts to peritoneal exudate cells from the donor strain. This group showed that cultured allografts were not susceptible to nonspecific immunostimulation or antibody and complement as administration of Freund's adjuvant (173) or anti-donor alloantiserum, with or without complement (207), failed to trigger islet allograft rejection.

Additional support for the passenger leukocyte concept is provided by studies reporting the disappearance of capillary endothelium, nerve endings and lymphoid cells by histological examination of rat and mouse islets following 7-day culture periods at 25°C or 37°C (135,192,208). Rabinovitch et al. (135) reported that rat islets which exhibited Ia antigen on intra-islet lymphocytes, macrophages and capillary endothelium, bound 45% less Ia antibody in a radioligand binding assay after a 7-day culture period at 37°C. This culture period reduced but did not eliminate Ia^+ cells. This reduction would likely account for the beneficial effect of short-term immunosuppression in conjunction with culture that was observed in some models (152-154,202). Gores et al. (138) recently reported that the removal of Ia^+-cells per se does not prevent rejection in congenic mouse strains and that a mechanism other than the elimination of these cells is responsible for the

reduction of tissue immunogenicity following culture. Nevertheless, culture with or without short-term immunosuppression can prolong allo- or xenograft survival depending on the strain combinations used.

(B) Pretreatment with Ia antisera

Because they had previously demonstrated that pancreatic β-cells lacked Ia antigen (131), Faustman et al. (209) attempted to eliminate Ia^+ APC and thus, prevent murine islet allograft rejection across a major histocompatibility barrier by treating islets with donor-specific Ia antisera and complement prior to transplantation. They found that briefly incubating islets with haplotype-specific Ia antibody plus complement resulted in 100% survival 200 days after intraportal transplantation. Recipient immunosuppression was not necessary and graft rejection could be induced by donor splenocytes, however, 20% of recipients receiving untreated islets also remained normoglycemic for 200 days. Nevertheless this report demonstrated that the passenger leukocytes thought to trigger the rejection response were Ia-bearing cells. In a later report, Faustman et al. (210) pretreated mouse islets with monoclonal dendritic cell antibody and complement and obtained 89% allograft survival at 65 days post-transplant, at which time four mice received donor dendritic cells and rejected their grafts. Four other mice were still normoglycemic at 200 days. Gores et al. (138) treated mouse islets with anti-Ia serum and complement or monoclonal anti-Ia antibody plus complement which, respectively, eliminated or reduced intra-islet Ia^+-cells as demonstrated by indirect immunofluorescence staining. Using the same strain combinations as Faustman et al. (209), they found that the survival of renal

subcapsular islet allografts in which Ia^+-cells were reduced (mean day of rejection; 15.8) was not significantly different from allografts in which Ia^+-cells were eliminated (mean day of rejection; 24.4). Neither pretreatment regimen prolonged survival over untreated islets allografts (mean day of rejection; 15.1). Gores et al. (138) concluded that the removal of donor Ia^+-cells per se does not prolong islet allograft survival but that the site of implantation (liver versus renal subcapsular space) may have a greater effect.

In a rat allograft model, Reece-Smith et al. (211) pretreated islets with mouse monoclonal antibodies against rat Ia-antigens plus guinea pig complement but did not observe significantly longer survival than control islets following renal subcapsular implantation. Terasaka et al. (202) also could not prolong rat islet allograft survival with cross-reacting mouse anti-class II antibodies but demonstrated 83% survival at 60 days when anti-Ia antibody-treated islets were intraportally transplanted to recipients receiving 30 mg/kg CyA for 3 days. Remarkably, Alejandro et al (212) have shown that the combination of anti-Ia monoclonal antibodies and low dose CyA can prolong islet allograft survival in outbred pancreatectomized dogs.

Recently anti-Ia immunotoxins, consisting of highly toxic ricin proteins coupled to monoclonal Ia antibodies, have been used in vitro to deplete islets of their passenger leukocyte population (213). A mixed lymphocyte/islet culture (MLIC) assay in which islets, rather than lymphocytes, function as the stimulator population was used to assess islet immunogenicity (213). This study demonstrated that the in vitro response of lymphocytes to allogeneic rat islets was eliminated, in a dose-dependent fashion, by pretreatment with the anti-Ia

immunotoxin and that this treatment had no effect on insulin secretion in response to glucose and theophylline. The effect of this pretreatment regimen on the immunogenicity of islets in vivo will be of great interest. Another unique way of eliminating Ia^+-cells is to dissociate pancreatic islets into single-cell suspensions and reassociate endocrine cells in culture, excluding lymphoid cells bearing Ia-antigen. Allotransplantation of these "pseudo" or neoislets resulted in 71% survival at 60 days and 100% survival at 60 days when recipients received a three day course of CyA (214).

The data from these investigators and the study by Morrow et al. (143) in which class II antigenic differences were eliminated genetically, suggest that the removal of the Ia-antigen itself will not eliminate islet immunogenicity but that the destruction or inactivation of the APC which happens to express Ia and can trigger the immune response by presenting class I antigens is necessary.

(C) Pretreatment with ultraviolet irradiation

Another method of minimizing tissue immunogenicity stems from studies which showed that lymphocytes or dendritic cells fail to proliferate in an allogeneic mixed leukocyte culture if they are exposed to ultraviolet irradiation (215). Thus, the effect of UVB irradiation is due to the inactivation of metabolically active Ia-antigen bearing cells rather than the depletion of these cells from the donor tissue. Inactivation of intra-islet leukocytes by direct UVB irradiation resulted in rat allograft survival for more than 250 days in the absence of immunosuppressive agents (216). When this study was extended to a strain classified as a high responder to donor

alloantigens, UVB-irradiated islets were rejected within one week of transplantation and cyclosporin alone (15 or 30 mg/kg/day) given at 0, 1, and 2 days after transplantation was ineffective in prolonging the survival of untreated islets in this strain combination (217). However, allograft survival was either markedly (mean survival of 18 days) or indefinitely prolonged (100% survival at 120 days) when direct UVB irradiation of islets was combined with a peri-transplant course of 15 or 30 mg/kg CyA, respectively (217).

This group has also shown that, in a xenograft model, indefinite survival of UVB-irradiated rat islets occurred in 6 of 8 mice when the mice were low responders (218). In a high responder strain, UVB irradiated rat islets survived for 30 to 40 days in 5 of 7 mice and for more than 70 days in the other two mice. In contrast, untreated rat islets were rejected in 6 to 8 days. In widely discordant species (primate-to-mouse), ALS and UVB irradiation were shown to have a synergistic effect on islet xenograft survival (219). Mean survival time was 19 days compared to 6 days with ALS alone. The effect of UVB irradiation in vivo appears to be dependent on the magnitude of in vitro mixed leukocyte cultures between donor and recipient strains. This method of reducing islet immunogenicity must be shown to be selective enough for clinical use.

In summary, the most successful attempts to prevent islet graft rejection have been based on the passenger leukocyte concept in which the intra-islet APC presents antigen and provides the second signal required for lymphocyte activation and the triggering of the immune response. Studies of islet allo- and xenograft survival by the groups

of Lacy, Lafferty and Hardy demonstrate that a reduction in the number of viable APC within the donor tissue results in reduced immunogenicity. Methods which selectively alter the function of passenger leukocytes, yet maintain islet viability are more effective in mice than rats and their effectiveness will depend on the donor-recipient strain combinations used. Thus, pretreatment of islets with culture in high O_2 or at low temperatures, anti-Ia or dendritic cell antibody plus complement, or UVB irradiation can destroy or inactivate APC but this treatment alone can not prevent rejection in rat recipients of strong immune responsiveness. When used in combination with short-term immunosuppression with ALS or CyA, however, islet allograft rejection can be prevented across major histocompatibility barriers.

HYPOTHESIS

Recently, a new method of minimizing islet immunogenicity by pretreatment prior to transplantation has been suggested. Like culture, anti-Ia antisera or UVB irradiation, cryopreservation has the potential of destroying or inactivating intra-islet APC without affecting endocrine cell viability and may, therefore, reduce islet immunogenicity. This hypothesis is based on the well-known differential susceptibility of various cell types to a freeze-thaw process. Mazur (100) has demonstrated that for all cell types, maximum cell survival occurs at an optimum cooling rate and optimum rates for diverse cell types vary greatly. Reported optimum cooling rates in the absence of cryoprotectant ranged from 1.6°C/min for mouse marrow stem cells to 7°C/min for yeast and 3000°C/min for human red blood cells

(100). Optimal cooling rates may also vary for more closely related cell types (220-223). In 1967, Bouroncle (220) reported that under the cryobiological conditions tested, different leukocyte populations were not preserved equally well. Farrant et al. (221-223) used cryopreservation to select for different leukocyte subpopulations, and, by varying cooling rates, could select between activated and non-activated peripheral blood lymphocytes (221). Others have examined the effect of cryopreservation on various subsets of peripheral blood mononuclear cells and concluded that cooling rate has different effects on subsets of these immunoregulatory cell types (224-228). T and B-lymphocytes, for example, are differentially damaged by freezing and thawing (224-226).

Because even closely related cells can be selectively destroyed by a particular cryopreservation protocol, it is conceivable that islet immunogenicity can be reduced if a protocol which is selective for islet function and destructive to passenger leukocytes is developed. Islet viability has been demonstrated in vitro following cryopreservation with a wide range of cooling rates and in vivo using slow cooling rates. Similarly, a variety of freezing protocols have been used to recover leukocytes and viability must be assessed carefully (227). Optimum conditions for lymphocytes include 7.5% to 15% DMSO, cooling at approximately 1°C/min with rapid thawing and removal of the additive by slow dilution at room temperature (227). Few studies have focussed directly on macrophage or dendritic cell cryopreservation. Several investigators have, however, reported a reduction in the number of macrophages following cryopreservation of human peripheral blood monocytes (228-231). Hem and Murthe-Kaas (232)

found that the protocol optimal for lymphocytes was not optimal for macrophages. Foreman et al. (233) tested cooling rates from 0.3 to 10°C/min and 800°C/min on mouse peritoneal macrophages. Using 5% DMSO, they found the optimum cooling rate was 1°C/min with rapid thawing. Sixty-three percent of human monocytes were recovered after freezing in 10% DMSO at 1.4°C/min to -30°C and rapid thawing (234). This study reported alterations in membrane-binding characteristics and mitochondrial structure, as well as a reduced chemotactic response. Taylor et al. (99) have recently examined lymphocyte and macrophage membrane integrity following a number of cryopreservation procedures which have been successful for islets. Optimum survival for these cell types occurred at cooling rates between 0.3 and 5°C/min. Destruction of lymphocytes and macrophages occurred at cooling rates greater than 75°C/min.

Because the critical cryobiological conditions used for successful preservation of lymphoid cells often differ from those used for the preservation of islets, these cell types may be differentially susceptible to a freeze-thaw protocol. Therefore, the hypothesis that cryopreservation can reduce islet immunogenicity was tested in rodent allo- and xenograft models using a cryopreservation protocol which is known to yield islets that are functional in vivo.

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II

REVERSAL OF DIABETES BY TRANSPLANTATION OF CRYOPRESERVED RAT ISLETS OF LANGERHANS TO THE RENAL SUBCAPSULAR SPACE[1]

The feasibility of transplanting freshly isolated islets of Langerhans to reverse experimentally-induced diabetes in rats is apparent (1) and in the last few years much progress has been made towards clinical application of this technique. Cryopreservation of pancreatic islets would offer several advantages to a transplant program by allowing the collection of islets from several donors, time for the selection and preparation of an appropriate recipient or modification of tissue immunogenicity, and a means for effective transport between centers (2).

Cryopreservation of isolated mouse or rat islets, using different cooling and warming rates or exposure to the cryoprotectant and various methods for the post-thaw removal of the cryoprotective additive, has yielded viable tissue. All of these variables are critical for cell recovery after freezing (3). Optimal recovery of islets has been reported using cooling rates ranging from 0.25 (2,4,5,16,17) to 1000°C/min (6). Post-thaw viability has been estimated by glucose or theophylline stimulated insulin release via static incubation or perifusion (2,6-17), the ability to synthesize proinsulin and DNA replication (9-13). Of these *in vitro* viability tests, perifusion appears to be the most sensitive (11), but the ultimate test of islet

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function is the ability to reverse experimentally induced diabetes following transplantation. Several laboratories demonstrating high survival *in vitro*, have had limited success *in vivo* (10,13,16). Since the initial work of Rajotte *et al.* (15), islets, slowly cooled in conjunction with a rapid thaw, have ameliorated the diabetic state in rats following intraportal transplantation (2,4,5). Similar success has been reported by Bretzel *et al.* (8,9). Unfortunately, removal of the grafted islets after transplantation to the liver is impossible. It is, therefore, difficult to prove that the reversal of diabetes was due to the transplanted islets and not a result of spontaneous β-cell recovery that has previously been reported following islet transplantation (18).

Islet transplantation beneath the kidney capsule allows removal of the graft and thus, can provide proof that the transplanted islets are responsible for normalizing the diabetic state. Other advantages include the ease of implantation and recovery by nephrectomy for histological purposes, as well as a somewhat immunoprivileged status in islet allograft survival (19). This site, however, does not fulfill the criteria for drainage to the portal circulation which, from a metabolic point of view, allows the islet's regulatory hormones to achieve their maximal effect (20,21). Cryopreserved fetal mouse proislets, transplanted beneath the kidney capsule of syngeneic recipients, were viable on the basis of immunocytochemical localization of insulin and glucagon at 5 weeks postimplantation (22). Only frozen-thawed fetal pancreas has been implanted beneath the kidney capsule of adult rats and shown to ameliorate streptozotocin (SZ) induced diabetes, provided it had first been grown for 21 days in a normoglycemic carrier (23).

In the present study, isolated rat islets of Langerhans are cryopreserved using the established protocol of Rajotte et al. (5) and implanted beneath the kidney capsule to determine their ability to reverse the course of streptozotocin induced diabetes from this site.

MATERIALS AND METHODS (Refer to Appendix D for details in full)

Islet Isolation. Islets were harvested from inbred Wistar-Furth (WF) rats as previously described (5). Following collagenase digestion and Ficoll purification, islets were washed 3 times with medium 199 supplemented with 10% fetal calf serum (v/v), penicillin (100 U/mL), and streptomycin (100 μg/mL), and then transferred to a petri dish. With the aid of a stereomicroscope, a modified reflected green light technique (24), and a finely drawn siliconized Pasteur pipette, known numbers of islets 100μm - 500μm were handpicked free of exocrine contamination.

Freezing Procedure. To minimize osmotic stress which may result from the addition of a hyperosmotic cryoprotectant, 2 M dimethyl sulfoxide (DMSO) was added to lots of 1000 islets in the step-wise fashion previously described by Rajotte et al. (5). The samples were transferred to a -7.3°C ethanol seeding bath for 5 min at which time nucleation was induced. After allowing for the release of the latent heat of fusion, the samples were transferred to an evacuated freezing Dewar flask containing a calibrated volume of 95% ethanol. Controlled cooling at 0.25°C was used from -10°C to -40°C after which the samples were transferred to liquid nitrogen for storage at -196°C.

Thawing and Removal of the Protective Additive. Following a storage period from 1 day to 4 weeks, the samples were thawed rapidly to 0°C in

a 37°C water bath at a rate of 200°C/min. The supernatant aspirated and 1.0 mL of 0.75 M sucrose was added at 0°C for a 30 min period. The sucrose was diluted at 25°C by addition of isotonic media at 5 min intervals as previously reported (5).

Transplantation. WF rats were individually housed in metabolic cages and nonfasting plasma glucose (PG,mg/dL), urine volume (UV,mL/24h) and urine glucose (UG,g/24h) were monitored weekly. After baseline values were obtained, diabetes was induced with SZ (Upjohn, Kalamazoo, Michigan), 60 mg per kg body wt injected via the penile vein. After 3 consecutive weekly PG values ≥300 mg/dL, 3000 freshly isolated (n=6) or cryopreserved (n=7) islets were implanted beneath the kidney capsule as follows: Recipients were anesthetized with 40 mg/kg sodium pentobarbital and 15-20 μL of blood from the recipient's tail was gently mixed with the islets and left to clot. The left kidney was exposed through a small flank incision and a fine glass rod, inserted through a small nick in the capsule, was used to separate the capsule from the kidney surface. The blood clot with embedded islets was inserted beneath the capsule using fine forceps. Recipients were monitored for all clinical indices daily for the first week post-transplant and weekly thereafter. PG and UG were determined by the glucose oxidase method (25) using a Beckman glucose analyzer.

Controls. Normal control rats received an injection of 0.1 M acetate buffer without SZ. Both normal and diabetic controls had sham transplants (a blood clot inserted beneath the kidney capsule) 3 wks following the induction of diabetes.

Glucose Tolerance Testing. Four months after transplantation, intravenous glucose tolerance tests (ivGTT) were performed on isograft

recipients and normal and diabetic control animals. Following an overnight fast, rats were anesthetized with 40 mg/kg sodium pentobarbital and a small incision was made over the femoral artery and vein. The femoral artery was isolated by blunt dissection and cannulated with polyethylene tubing (PE-50) attached to a three-way stopcock. Glucose (0.5g/kg) was injected via the penile vein and 0.6 mL blood samples were taken at 0, 1, 5, 10, 15, 60, and 90 min. Blood was collected in heparinized tubes and centrifuged immediately. The plasma was removed and assayed for glucose and insulin. Radioimmunoassay for insulin was by the double antibody technique (26) with Novo rat insulin standards (Lot No. R 8303082). K values (decline in PG concentration, %/min) were calculated from 5, 10, 15, and 30 min PG values.

Nephrectomy and Histology. At varying periods following the ivGTT, the graft-bearing kidney was removed and the clinical indices were monitored for 1 wk. The kidney was fixed in aqueous Bouin's and sectioned at the graft site. Peroxidase antiperoxidase staining for insulin (27) was performed on these tissue sections.

Statistical analysis. Results are expressed as mean ± standard error of the mean (SEM). Unpaired Student t-tests were used to compare groups at all time points as well as ivGTT K values. The criterion for statistical significance was $P \leq 0.05$.

RESULTS

Figure II-1 compares the clinical responses of diabetic rats before and after transplantation of 2000 freshly isolated or cryopreserved islets beneath the kidney capsule. The degree of

SZ-induced diabetes did not differ in any of the groups and spontaneous remission of the diabetic state was not observed in diabetic controls throughout the course of the study. Following transplantation, PG and UV returned to baseline values and glycosuria disappeared. UV was the most sensitive indicator of islet function, returning to baseline values within 3 days in recipients of fresh isografts and in 8 days in recipients of cryopreserved islets. PG and UG in recipients of fresh or frozen-thawed islets normalized in 4 and 10 days, respectively. After 1 wk post-transplant, clinical indices were not significantly different between these groups. Figure II-1 also demonstrates an immediate return of hyperglycemia, polyuria, and glycosuria in all islet recipients following removal of the graft-bearing kidney. In the fresh and cryopreserved isograft groups, respectively, all animals lost weight (15 vs 17g/wk) during the diabetic period, gained weight (6 vs 7g/wk) after transplantation, and showed an immediate loss of weight (47 vs 31g/wk) following nephrectomy. Table II-1 shows the mean values for PG, UV, and UG in the transplanted groups during the 2 wk baseline, 3 wk diabetic, 16 wk post-transplant, and post-nephrectomy periods. There were no significant differences between groups during these time periods.

Results of the 4 month ivGTT (0.5 g glucose/kg) for all groups of animals are shown in Figure II-2. Only at the 30 min time point did PG of animals transplanted with freshly isolated islets differ significantly ($0.05>P>0.02$) from animals that received frozen-thawed islets. Mean K values for these two groups (1.68 ± 0.34%/min and 1.20 ± 0.16%/min, respectively) were not significantly different. Normal and diabetic control K values were 2.60 ± 0.31%/min and 0.61 ± 0.12%/min,

respectively. In response to the glucose load, peripheral plasma insulin levels (mU/L) in recipients of fresh and cryopreserved islets showed a typical biphasic insulin release pattern and were not significantly different at any point during the test.

Macroscopic examination of graft-bearing kidneys showed diffuse areas of white tissue with small blood vessels visible beneath the capsule. Histological examination revealed masses of endocrine tissue with well preserved morphology beneath the renal capsule. Plate II-1 shows immunoperoxidase stained kidney sections with abundant quantities of insulin localized within islet tissue implanted immediately after isolation (A) or after cryopreservation (B).

DISCUSSION

Isolated rodent islets of Langerhans have been cryopreserved by protocols which differ greatly in the pre-freeze, cooling, warming, or post-thaw conditions. Reports on islet cryopreservation also differ in the way viability is assessed. Using a brief period of exposure to 1 M DMSO and a cooling rate of 75°C/min, Bank et al. reported high survival of rat islets on the basis of net insulin release after glucose challenge (7). Others have found that in accordance with the theoretical calculations of Mazur (3), slower cooling rates provide maximum retention of viability (2,4,5,8-10,16,17). Andersson and Sandler, using cooling rates of 0.5 or 5.0°C/min (10-12), and more recently 25°C/min (13), preserved mouse islet function by a variety of in vitro assays but had limited success in vivo. Taylor and Benton have recently reported that islets exposed to increasing concentrations of DMSO for 35 min showed no difference in survival (net insulin

production during static incubation) whether they were cooled at 0.3 or 1000°C/min (6). This apparent insensitivity to cooling rate may be in their interpretation of net insulin production. Care must be taken in using post-stimulation insulin release to calculate viability as we and others (14,16,17) have found that frozen-thawed islets lose some responsiveness to low glucose and are unable to return to basal rates of insulin release once glucose stimulation is stopped. Caution must be taken in the assessment of islet viability with *in vitro* techniques in that some groups reporting high survival *in vitro* have had difficulty demonstrating function *in vivo* (10,13,16). Taylor *et al.* (16) showed that intraportal transplantation of islets, slowly cooled in 2 M or 3 M DMSO, normalized 2 of 5 and 1 of 3 rats, respectively. Sandler *et al.* (13) reversed alloxan-diabetes completely in 3 and partially in 4 of 9 mice by intrasplenic transplantation of 600-800 syngeneic islets, frozen at 25°C/min. However, Bretzel (8,9), using islets frozen at 2°C/min to -30°C, followed by 5°C/min to -100°C, and Rajotte (2,4,5), by slow cooling at 0.25°C/min to -40°C, achieved much better results with intraportal transplantation as demonstrated by long-term normalization of plasma glucose, urine volume and urine glucose in diabetic recipients. Rajotte's protocol, with a higher temperature for the final equilibration in DMSO, has been applied to canine pancreatic fragments and human isolated islets and shown to yield viable tissue both *in vitro* (28) and *in vivo* (4,5,29-31).

The present study has shown that cryopreserved islets of Langerhans can reverse SZ-induced diabetes in rats following transplantation beneath the kidney capsule. The diabetic state of rats was promptly reversed following implantation of either fresh or

cryopreserved islet isografts. The clinical indices of recipients of freshly isolated islets reached pre-transplant values within the first week of transplantation whereas islets that had been cryopreserved prior to transplantation produced a similar response in recipients by the second week post-transplant. Based on a 90% recovery (unpublished data from our laboratory), approximately 2700 cryopreserved islets were transplanted compared to 3000 fresh so the longer time required for cryopreserved grafts to reverse the diabetes may be a reflection of the smaller mass transplanted. Cryopreserved islets may also suffer some minor ultrastructural damage which is reversible as some investigators have found a post-thaw culture period is beneficial (2,12). Thus minor freezing injuries and cellular repair during the first days following transplantation of cryopreserved islets may also account for the delay in attaining normoglycemia.

Because of the lack of portal drainage from the renal subcapsular space, we thought that greater numbers of islets would be necessary to achieve the same clinical effect as 3000 islets transplanted to the liver (4,5). In this study, 3000 islets implanted beneath the kidney capsule effectively normalized the animals. This report demonstrates no significant difference in mean K values of fresh and frozen-thawed islet isograft groups at the 4 month ivGTT. This is in contrast to previous studies in which recipients of cryopreserved islet isografts had significantly different K values from control animals bearing fresh isografts at 3 months post-transplant (2,4,5,16). Removal of the graft-bearing kidney 16 weeks after islet implantation proved conclusively that the transplanted islets were responsible for the normalization of hyperglycemia, polyuria, and glycosuria as these

characteristics of the diabetic state returned immediately after nephrectomy.

Islets cryopreserved by various protocols and shown to function *in vitro* must also demonstrate function *in vivo*. Using slow cooling to -40°C in combination with rapid thawing, cryopreserved rat islets of Langerhans transplanted beneath the kidney capsule can normalize diabetic recipients as well as freshly isolated islets. Successful cryopreservation of islet tissue will facilitate the clinical application of pancreatic islet cell transplantation.

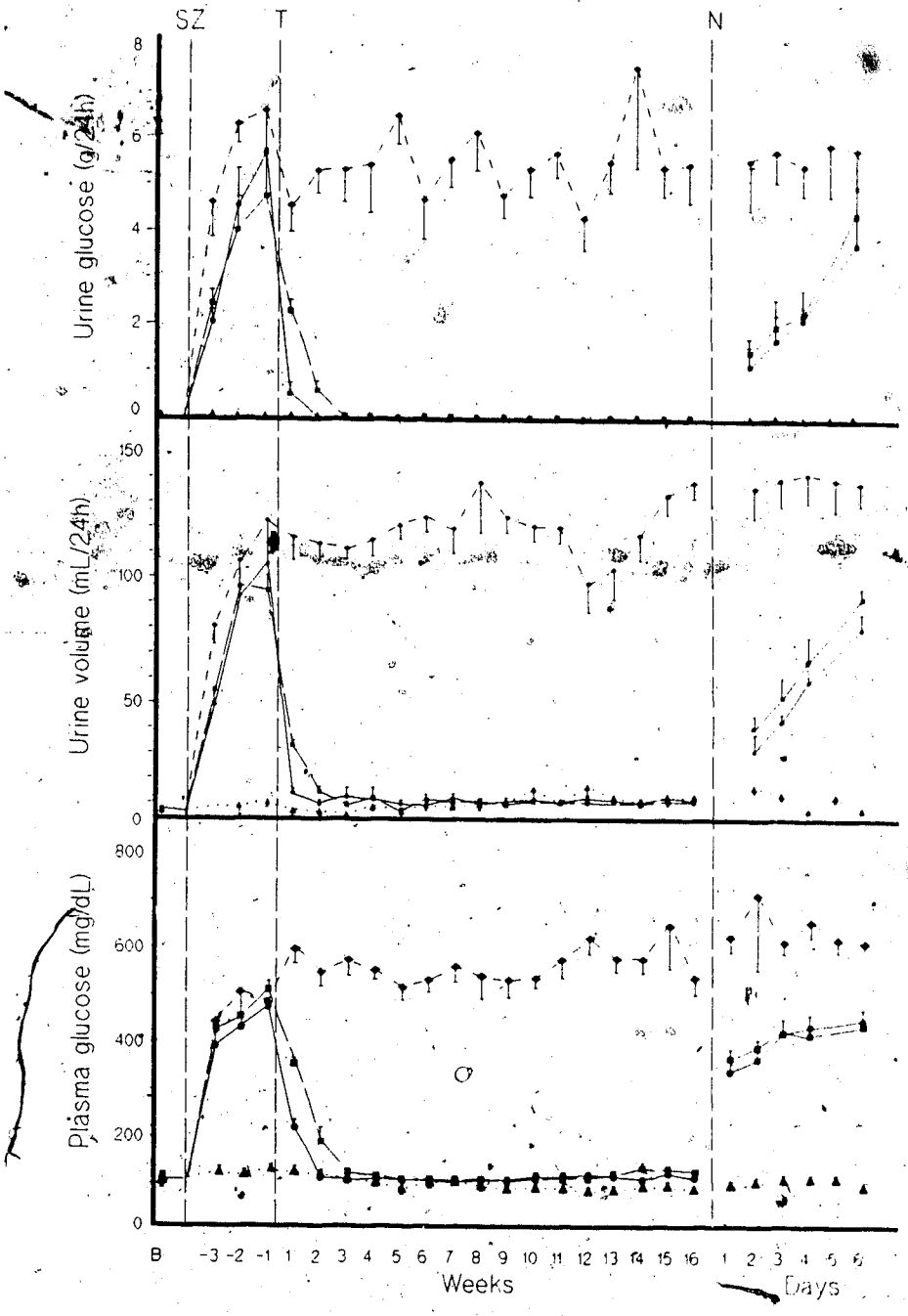

FIGURE II-1. Clinical responses of diabetic rats after syngeneic islet-transplantation. 3000 handpicked islets were implanted beneath the kidney capsule immediately after isolation (n=6 •—•) or after cryopreservation (n=7 •---•). Clinical indices of sham-transplanted normal (n=4 ▲ ▲) and diabetic (n=8 •···•) controls are shown for comparison. B - baseline, SZ - streptozotocin, T - transplantation, N - nephrectomy.

TABLE II-1. Clinical responses* in SZ-induced diabetic rats transplanted with 3000 fresh or cryopreserved islets.

GROUP	INDEX	BASELINE	DIABETIC	POST TRANSPLANT	GRAFT NEPHRECTOMY	ivGTT K VALUE+
FRESH ‡	PG	111.5± 3.8	434.0±13.4	118.9± 6.7	395.1±14.5	1.68±.34
	UV	4.6± 0.6	82.4± 8.3	11.0± 0.3	51.6± 4.9	
	UG	0.002±.0003	4.1± 0.5	0.03± .03	2.1± 0.3	
CRYO ‡	PG	113.6± 2.7	460.8±14.1	137.1±16.1	402.8±11.0	1.20±.17
	UV	6.1± 0.5	82.7± 5.9	11.8± 1.4	51.8± 5.9	
	UG	0.004±0.001	3.8± 0.4	0.2± 0.2	2.3± 0.3	

* Mean (±SEM) values of plasma glucose (PG mg/dL), urine volume (UV mL/24h) and urine glucose (UG g/24h) during the baseline, diabetic, post-transplant and post-nephrectomy periods.

+ Mean (±SEM) K values (decline in plasma glucose concentration, %/min) from intravenous glucose tolerance tests following islet transplantation.

‡ Fresh vs cryo, $p>0.05$ for all indices.

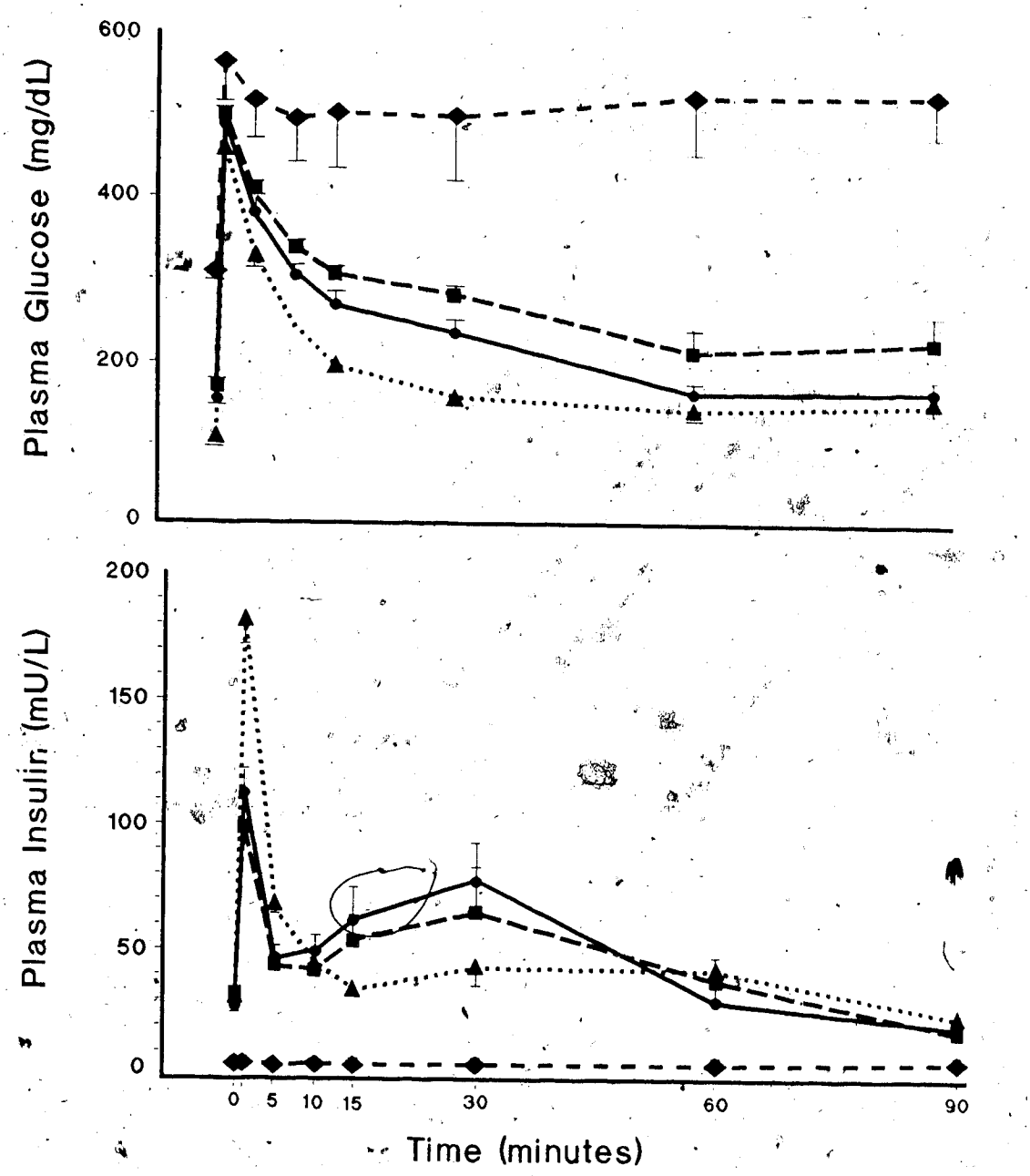

FIGURE II-2. Results of intravenous glucose tolerance tests 4 months after transplantation of 3000 freshly isolated (n=6 ●——●) or cryopreserved (n=7 ■——■) islets. Mean K values (decline in plasma glucose %/min) were not significantly different between these groups. There were no significant differences in the peripheral plasma insulin in these two groups. Normal (n=4 ▲·····▲) and diabetic controls (n=5 ◆——◆) are shown for comparison.

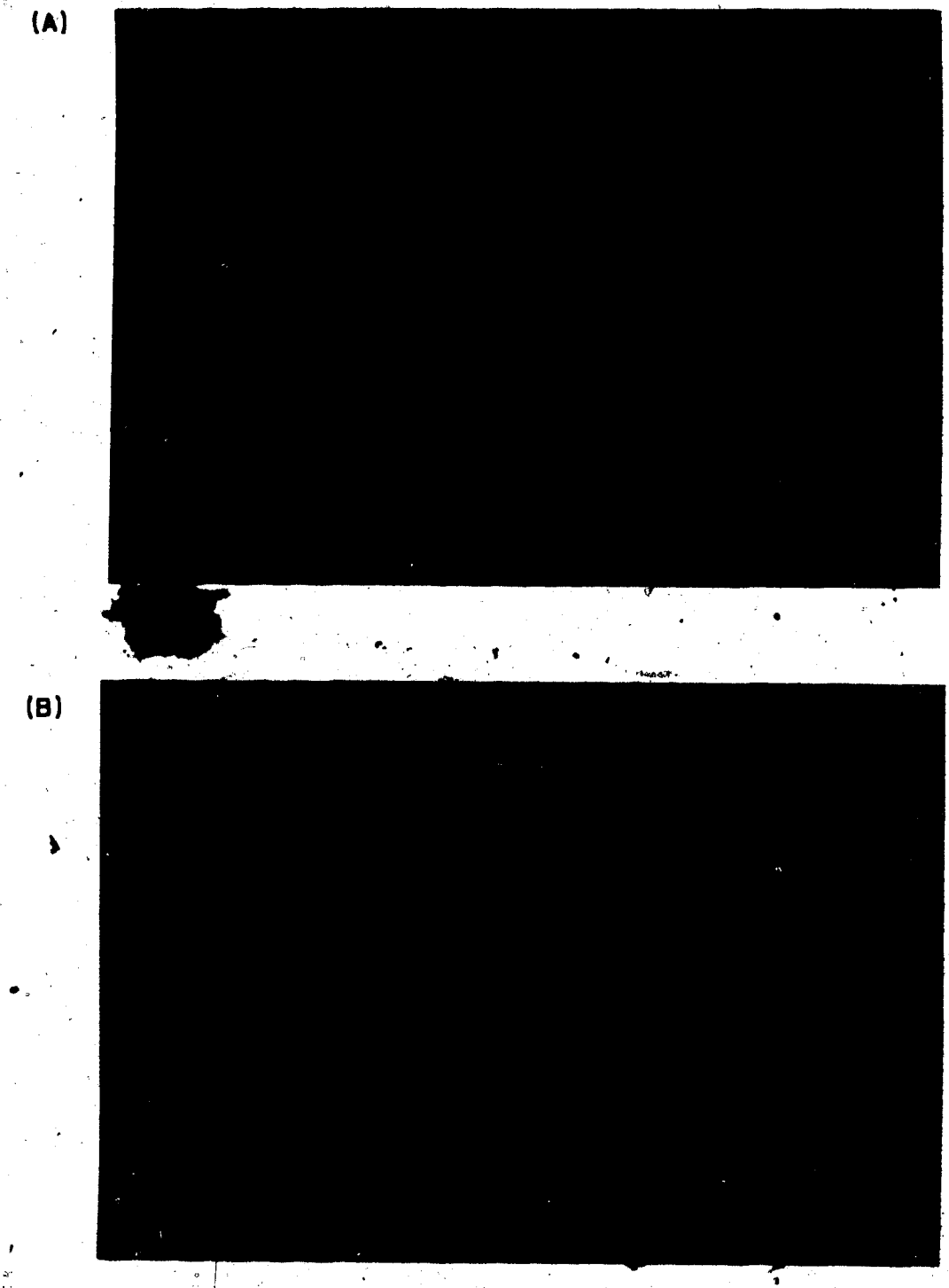

PLATE II-1. Histological appearance of freshly isolated **(A)** or cryopreserved **(B)** rat islets of Langerhans 17 weeks after implantation beneath the kidney capsule. Immunoperoxidase staining for insulin revealed well granulated β-cells.

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III

PROLONGATION OF ISLET XENOGRAFT SURVIVAL BY CRYOPRESERVATION[1]

Pretreatment regimens such as *in vitro* culture (1,2), anti-Ia serum plus complement (3), or ultraviolet irradiation (4) have successfully prevented the rejection of rodent pancreatic islet allografts. The survival of concordant (rat to mouse) islet xenografts has been markedly prolonged by low temperature culture (24°C) in conjunction with short-term anti-lymphocyte serum (ALS) (5) or cyclosporin (6), or 37°C culture of rat megaislets in 95% O_2 (7). These procedures are aimed at the selective elimination of immunostimulatory antigen-presenting cells, an approach based on the concept that passenger leukocytes residing in donor tissue provide the major barrier to successful tissue transplantation (8).

Cryopreservation of isolated islets or pancreatic fragments has yielded viable tissue that can ameliorate experimentally induced diabetes in rodents and larger animals (9-11). Because distinct cell types are known to be differentially susceptible to a particular freeze-thaw process (12), it is conceivable that islet immunogenicity may be modulated with a cryobiological approach. We examined this possibility in a xenograft model by a cryopreservation protocol known to yield islets that are functional *in vivo*.

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[1] A version of this chapter has been accepted for publication. Coulombe MG, Warnock GL, Rajotte RV. 1987. Diabetes 36(9).

MATERIALS AND METHODS (Refer to Appendix D for details in full)

Animals. Male Wistar-Furth rats (Harlan Sprague Dawley) were used as islet donors. Recipients were male BALB/c mice obtained locally. The mice were individually housed in metabolic cages, and baseline nonfasting plasma glucose (PG), urine volume, urine glucose, and weight were monitored. Mice were anesthetized with tribromoethanol (Aldrich, Milwaukee, WI) dissolved in tert-amyl alcohol (Fisher Scientific) (0.01 mL/g) (13), and were rendered diabetic with streptozotocin 200 mg/kg body wt via the retroorbital sinus. Clinical indices were monitored three times over two wk before transplantation.

Isolation and cryopreservation of islet tissue. Islets were isolated by collagenase digestion and Ficoll purification and were meticulously handpicked free of exocrine contamination as previously described (9). Aliquots containing 500 islets ≥100 μm were cryopreserved or immediately transplanted.

The freeze-thaw sequence used was that of Rajotte et al. (10). Briefly, dimethyl sulfoxide (DMSO) was added stepwise to a final concentration of 2 M. The samples were supercooled to -7.3°C, nucleated, slowly cooled at 0.25°C/min to -40°C, then quickly frozen to -196°C for storage. The samples were rapidly thawed (200°C/min) in a 37°C water bath, and the hyperosmolar DMSO was removed with 0.75 M sucrose at 0°C. The sucrose was diluted with stepwise additions of isotonic media at 25°C.

Transplantation. In all cases, 500 islets were embedded in a blood clot and inserted beneath the kidney capsule of recipient mice having PG values >500 mg/dL. Mice in group 1 and group 2 received freshly isolated or frozen-thawed islets, respectively. Mice in groups 3 and 4

received fresh or cryopreserved islets, respectively, in conjunction with a single injection of rabbit anti-mouse lymphocyte serum (0.2 mL) and rabbit anti-rat lymphocyte serum (0.1 mL) administered intravenously 5 min before transplantation. All clinical indices were monitored daily until rejection (defined as the first of 3 consecutive days of plasma glucose $\geq$ 200 mg/dL) was evident.

RESULTS

In each group, all clinical indices returned to baseline values within 2-4 days after islet transplantation. Once graft rejection was initiated, it was extremely rapid, reaching pretransplant diabetic values within 5 days. As shown in Table III-1, freshly isolated rat islets implanted beneath the kidney capsule of diabetic mice (Group 1) were rejected in 10-13 days, and cryopreserved islets (Group 2) were rejected in 8-17 days. When ALS was administered, graft survival was extended to 13-26 days in the fresh and 16-37 days in the cryopreserved groups. Analysis by the Jonckheere Terpstra test for ordered alternatives (14) showed that treatment groups 1-4 had an increasing effect on extending xenograft survival, and this was highly significant. The effect is demonstrated in Figure III-1. Beyond 10 days after transplantation, the groups of mice that received cryopreserved islets, with or without ALS, showed greater proportions of surviving grafts than their fresh counterparts. Twelve days after transplantation, 27.3% of fresh islet xenografts and 50.0% of cryopreserved grafts were surviving. After 13 days, all fresh xenografts had rejected, but 37.5% of cryopreserved grafts were still functioning. When ALS was given, 54.5% of cryopreserved grafts and

6.2% of fresh grafts were functioning 19 days after transplantation. After 26 days, when all fresh grafts had rejected, 27.3% of frozen-thawed grafts were still able to normalize the animals.

DISCUSSION

Cooling rates optimal for cell survival can vary widely for different cell types (12). Thus, a given cryopreservation protocol can selectively destroy cells of one type and preserve the function of another type. Cryopreservation of canine pancreatic fragments can simultaneously destroy exocrine tissue and preserve endocrine function (9,15). This differential susceptibility of various cell types to freeze-thaw damage was tested on isolated rat islets in an attempt to selectively destroy or inactivate immunocompetent passenger leukocytes. Our results indicate that cryopreservation can reduce islet immunogenicity. The survival of 500 fresh rat islets inserted beneath the kidney capsule of nonimmunosuppressed mice compares with that reported by Bobzien et al. (16). A greater percentage of cryopreserved islet xenografts, with or without ALS, showed extended graft survival; however, survival was only moderately prolonged compared with cultured islets (5-7) or cryopreserved islet allografts (17). Although this extended xenograft survival may be a result of exocrine tissue destruction, it appears the cryopreservation protocol does eliminate or inactivate passenger leukocytes from the islet graft because the islets were carefully handpicked free of exocrine tissue. Some passenger leukocytes, however, may have survived. Taylor et al. (18) have shown that lymphocytes and macrophages are more susceptible to damage at fast cooling rates (75°C/min). Further investigations should include

detection of Ia-positive cells within the islets before and after freezing and examinations of other freezing protocols that will achieve maximum destruction of immunostimulatory antigen-presenting cells without affecting endocrine function. The ability of cryopreservation to reduce islet immunogenicity and establish a low-temperature tissue bank could have important applications to clinical islet cell transplantation.

TABLE III-1. Effect of cryopreservation and anti-lymphocyte serum on islet xenograft survival (rat-to-mouse).

Group	Treatment (T)	n	Day of Rejection*
1	Fresh (control)	11	10, 10, 11, 11, 11, 11, 12, 12, 13, 13, 13
2	Cryopreservation	8	8, 10, 11, 11, 13, 14, 16, 17
3	Fresh + ALS+	16	13, 14, 15, 15, 15, 16, 16, 17, 17, 18, 18, 19, 19, 19, 19, 26
4	Cryopreservation + ALS+	11	16, 16, 16, 17, 18, 20, 23, 23, 28, 29, 37

*Defined as the first of 3 consecutive days of plasma glucose ≥ 200 mg/dL

+Mice received one injection of rabbit anti-mouse and rabbit anti-rat lymphocyte serum (ALS) at the time of transplantation

Jonckheere Terpstra test for ordered alternatives: $J^* = 5.576 > z_{(.001)} = 3.090$
$T1 < T2 < T3 < T4$ significant at the 0.1% level

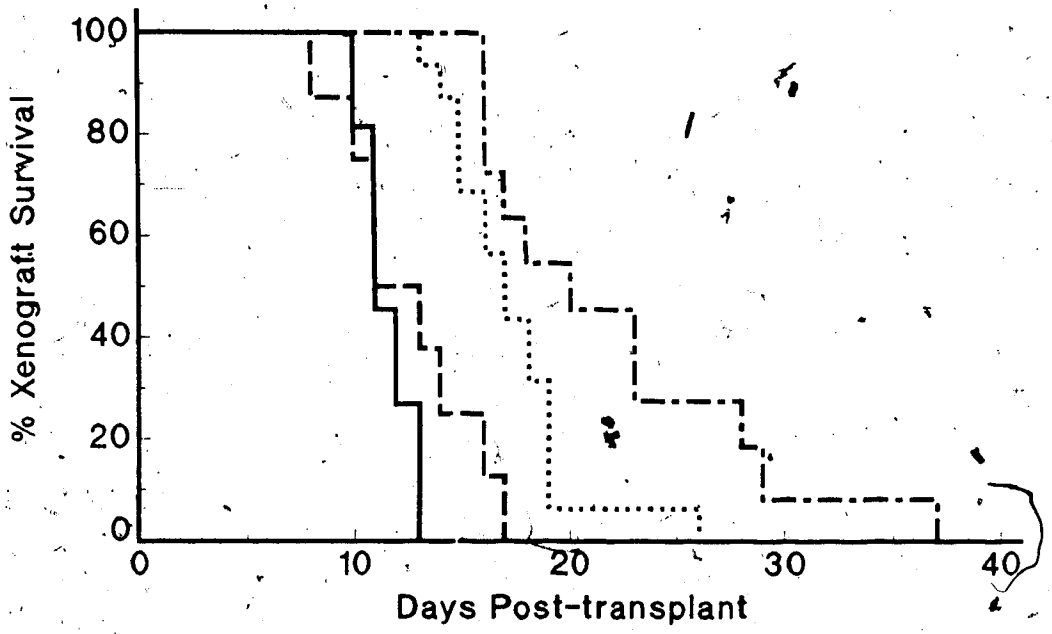

FIGURE III-1. Percent xenograft survival of fresh (———) or cryopreserved (– – –) islets and of fresh (·····) or cryopreserved (–·–·–) islets in conjunction with a single injection of ALS at the time of transplantation.

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IV

DISCUSSION

A common criticism of intraportal isologous islet transplantation studies has been that they cannot conclusively prove that the reversal of experimentally-induced diabetes is due solely to the transplanted islets. This criticism was supported by a 1984 report on the regeneration of pancreatic β-cells in SZ-induced diabetic rats following intraportal islet transplantation (1). The spontaneous remission of the diabetic state did not occur in non-transplanted animals and therefore, was likely triggered by the correction of the metabolic state. This phenomenon should be investigated further as it questions reports on the reversal of diabetes following isogeneic intraportal islet transplantation. The renal subcapsular site was chosen for this study because one can demonstrate that, by unilateral nephrectomy, grafted islets are entirely responsible for normoglycemia. This site was also advantageous in that transplantation was a less stressful procedure, particularly for diabetic mice, and histological examination of the graft site was easier. In addition, cryopreserved islets have only been transplanted to the liver via the portal vein.

ISOGRAFT STUDY

This study showed that cryopreserved islet isografts implanted beneath the kidney capsule could normalize the diabetic state long-term and could do so as well as freshly isolated islets. Following cryopreservation, most islets remained intact but some loss of viability is inevitable. Post-thaw correction for this loss was not made so all cryopreserved groups represent a smaller islet mass than their fresh counterparts. Nevertheless, at one week post-transplant,

clinical indices (PG, UV, UG, and WT) of recipients of fresh or cryopreserved isologous islets were not significantly different (see Figure II-1 and the plotted weight response in Appendix A, Figure A-1). Nonfasting PG, UV, and UG were all in normal range unlike the studies by Bretzel et al. (2,3) which reported altered postprandial glucose metabolism. As suggested by Bretzel, this may have been due to the small number of islets transplanted.

Four months after transplantation, ivGTT K values (1.68±0.34 %/min for fresh and 1.20±0.16 %/min for cryopreserved isograft recipients) were not significantly different. Previous reports from Rajotte's group (4-6) and Taylor et al. (7) have indicated that recipients of cryopreserved islets do not respond as well as recipients of fresh islets in ivGTT 3 months following intraportal islet transplantation. Unfortunately this procedure has not been reported by Bretzel's group. The normal control K value (2.60±0.31 %/min) is not directly comparable to that of the isograft recipients because the latter represent secretion of insulin into the systemic as compared to the portal circulation. Reece-Smith et al. (8) have shown that the K value following transplantation of isolated islets beneath the kidney capsule (1.7±0.5 %/min) could be increased to normal (2.5±0.4 %/min) after a renal portal shunt. Similar results with transplanted fetal rat pancreas have been reported by Brown et al. (9). Portal drainage of islet secretions is important in the physiologic control of glucose metabolism because the liver removes approximately half of the insulin delivered to it in a single passage of portal blood (9).

Figure II-2 shows the plasma insulin responses during ivGTT. The first insulin peak, representing stored insulin, was significantly

higher in normal controls than in isograft recipients. This is due to the larger number of islets in the pancreas and likely a larger proportion present in a granulated state. The second peak which represents newly synthesized insulin is higher in transplanted animals. This is necessary to cover the higher plasma glucose concentrations and may also be a result of insulin secretion into the systemic circulation. This would be consistent with the reports of Brown et al. (9) and Reece-Smith et al. (8). They showed a fall in IRI values after a renal portal shunt because insulin secreted into the portal circulation is extracted by the liver before reaching the peripheral blood.

Histological examination of the graft-bearing kidney four months after transplantation revealed well-granulated β-cells within the islets. Sections were stained with immunoperoxidase or aldehyde fuchsin. This isograft study conclusively showed that 3000 cryopreserved islets, independent of immunologic factors, can successfully reverse diabetes in WF rats following implantation beneath the kidney capsule. It also established a model for the allogeneic transplantation studies.

ALLOGRAFT STUDY

Many of the early reports on the unusual vulnerability of islets to rejection suggested that contaminating exocrine debris in islet preparations may be responsible for the increased immunogenicity of the graft. Crude islet preparations may contain small lymph nodes and vascular or ductal tissue in addition to exocrine debris. Some (10-12) but not all (13) investigators agree that impure islet preparations have an adverse effect on allograft acceptance. In addition, exocrine

tissue has been shown to adversely affect implantation of rat islets beneath the kidney capsule (14) and the insulin response to glucose challenge (15). For these reasons, "clean" islets, free from any contamination, were handpicked from the Ficoll preparation and then picked, sized and counted a second time to ensure islet purity in allo- and xenograft studies.

In an attempt to determine whether cryopreservation could reduce islet immunogenicity, fresh or cryopreserved WF islets were implanted beneath the kidney capsule of diabetic Lewis (LEW) recipients, with or without a short-course of cyclosporin. Lewis rats exhibit strong immune responsiveness to WF alloantigens. When WF islets are transplanted into LEW recipients, they are usually rejected in a mean of 5-7 days (16-19). Prolonged or indefinite acceptance of immunoaltered islet allografts across this major histocompatibility barrier has been reported. Culture at 24°C and an injection of ALS prolonged WF to LEW allograft survival indefinitely after either intraportal (16,17) or renal subcapsular (18) implantation. In the absence of ALS, cultured islets were rejected in 5-10 days. Lacy et al. (16) reported that two recipients given RALS and fresh islets had not rejected their grafts at 100 days. When islets were cultured in high O_2 alone, intraportal allograft survival in this strain combination was prolonged indefinitely (19). Not all investigators had such success. Rabinovitch et al. (20) reported a mean intraportal allograft survival time of 27 days following 7 day culture at 37°C and an injection of ALS. The rejection criteria in this study (the return of glycosuria, 8-14 g/day) was less stringent than the usual criteria of plasma glucose exceeding 200 mg/dL.

The short-term cyclosporin-treatment of LEW recipients was modelled after the studies of Lau et al. (21) and Terasaka et al. (12) in which CyA (30 mg/kg) was given on the day of islet transplantation as well as the first and second days after transplantation. Indefinite intraportal WF to LEW allograft survival was obtained when this short-term CyA therapy was used in combination with 24°C culture or Ia antibodies (12) or UVB irradiation (21). In this study, an additional injection of CyA was given subcutaneously on the day prior to transplantation so that trough levels were elevated at the time of transplantation.

Complete reversal of the diabetic state after implantation of 3000 fresh or cryopreserved /allogeneic islets was not observed indicating that rejection was initiated before normoglycemia was restored. Selected data (PG and UV responses) are shown in Appendix B. Because mean values for each group masked any effect of transplantation, individual responses are plotted. Plasma glucose responses, shown in Figure B-1 to B-4, were quite erratic in all recipients. The previously set criteria for rejection (plasma glucose ≥200 mg/dL) could not be met and clinical responses were not uniform enough to establish any reliable criteria for rejection, particularly since cryopreserved islets in an isograft model took a week longer to normalize the animals.

Urine volume was the most sensitive indicator of islet function. Responses are shown in Figures B-5 to B-8. The trend observed with CyA was that rejection was not as abrupt with cryopreserved islets as with fresh islets. Once rejection was initiated, all animals in the fresh with CyA group (Figure B-7) quickly reached pre-transplant levels. In

6 of 10 recipients of cryopreserved islets (Figure B-8), it took much longer to reach pre-transplant values. The selective destruction of Ia^+-cells by cryopreservation in a proportion of islets may explain the gradation in rejection times observed in this group. The islets able to evade rejection, however, are not present in sufficient numbers to normalize the animals and eventually die. All clinical indices showed the same trend.

Seven days after transplantation, histological examination of fresh or cryopreserved WF islets beneath the kidney capsule of LEW recipients revealed total lymphocytic infiltration and a lack of viable endocrine cells. Nephrectomies were performed on allograft recipients at 2 weeks post-transplant and at 6 weeks post-transplant in the animals which received peri-transplant CyA. With one exception, complete rejection had occurred prior to nephrectomy so the removal of the graft-bearing kidney did not exacerbate the diabetic state. The exception was a rat in the cryopreservation with CyA group. This animal showed evidence of function up to 6 weeks post-transplant after which there was a gradual increase in PG, UV, and UG. Nephrectomy after 8 weeks resulted in a sharper increase in PG, UV, and UG as well as a drop in weight. This animal lends support to the hypothesis that cryopreservation can reduce islet immunogenicity.

Although Reece-Smith *et al.* (22) have suggested that the kidney capsule is an immunoprivileged site, this has been refuted (23) and it is possible that transplanting islets to the liver via the portal vein may have yielded better results. This site may allow for quicker revascularization and thus a more rapid return to baseline values from which rejection could be assessed. Also brief peri-transplant

immunosuppression with ALS may have been more effective than CyA. ALS is a more potent immunosuppressive agent and may have been able to block the immune response long enough for revascularization of the graft and normoglycemia to occur.

XENOGRAFT STUDY

Greater success was attained using a rat-to-mouse xenograft model. As shown in Appendix C (Table C-1), the transplantation of 900 fresh WF rat islets beneath the kidney capsule of diabetic BALB/c mice resulted in death within 24 hours. This was likely due to hypoglycemia and thus, the number of islets transplanted was decreased to 700 and then to 500. This number in combination with a subcutaneous injection of dextrose at the time of transplantation increased survival post-transplant to almost 100%. ALS treatment seemed to increase post-transplant mortality.

BALB/c recipients received 500 fresh or cryopreserved rat islets, with or without a single injection of ALS at the time of transplantation. Clinical indices were monitored daily post-transplant and are shown in Appendix C, Figures C-2 to C-4. Table C-2 shows the mean plasma glucose concentrations during baseline monitoring, 1 week prior to and 1 week after transplantation. There were no significant differences between groups at these time points. Histological examination of the graft site revealed extensive lymphocytic infiltration at 6 but not 4 days post-transplant (Plate C-1).

ALS, by itself, prolonged the survival of both fresh and cryopreserved islets but the effect was moderate compared to the study of Lacy *et al*. (24) in which MALS alone, in the same donor-recipient strain combination, prolonged fresh graft survival from 8-49 days.

Cryopreservation alone prolonged survival although not as much as fresh grafts in combination with ALS. The combination of cryopreservation and ALS had the greatest effect on extending graft survival and like the reports by Lacy (12,25) and Hardy (21) with short-term CyA, this effect was synergistic. Unfortunately survival of cryopreserved xenografts was only moderately prolonged compared with cultured islets (24-27). In the same donor-recipient strain combination, the transplantation of islets cultured for 7 days at 24°C and the administration of ALS resulted in 70% survival at 100 days post-transplant (24). Megaislets cultured in 95% O_2, with or without ALS, survived greater than 50 days (26). Nevertheless, data in Figure III-1 and Table III-1 show that the cryopreservation protocol used in this study prolonged islet xenograft survival over fresh islet xenografts.

The hypothesis that cryopreservation can reduce islet immunogenicity was based on the principle that different cells require different freeze-thaw conditions. The assumption was that islet endocrine cells and the associated passenger leukocytes are differentially susceptible to the cryopreservation process. Although this appears to be true, the testing of this hypothesis is far from over. Leukocytes are usually frozen in 10% (1.3M) DMSO with 10-20% serum in an isotonic medium at 1°C/min to below -30°C or -40°C. They are usually thawed rapidly and the DMSO is slowly diluted with isotonic media at room temperature (28). This protocol is generally similar to that used for the cryopreservation of islets and because cryopreserved xenografts did not survive to the extent of cultured xenografts, it appears that some leukocytes are surviving the freeze-thaw process.

Thus, further studies are needed to determine which steps of the freeze-thaw procedure are most critical for the selective destruction of leukocytes and how these aspects can be incorporated into a new cryopreservation protocol without sacrificing islet viability.

Exposure to the cryoprotectant is one factor which may affect the susceptibility of endocrine cells and leukocytes to the freezing procedure. The optimal concentration of DMSO and the time and temperature at which the cells are exposed is a compromise between protection and toxicity. Toxic effects of DMSO on leukocytes have been reported (29-32). Hem (31) found the optimum concentration of DMSO for both rat spleen and lymph node cells to be 5-10% when the cooling rate was 1°C/min. He noted decreased survival when the concentration was 15% (1.9 M). Also at a cooling rate of 1°C/min, 5% (0.6 M) DMSO was optimum for mouse lymphocytes (30) and mouse peritoneal macrophages (32). Bouroncle (29) reported that the toxicity of DMSO was more pronounced for mature granulocytes and monocytes than for other leukocyte subpopulations. Exposure of leukocytes to DMSO at 20°C reduced survival compared to exposure at 0°C or 4°C (29,30). Closely related to the prefreeze permeation of the cryoprotectant is its removal post-thaw. Rapid dilution of the cryoprotectant after thawing or slow stepwise dilution at 0°C rather than room temperature resulted in poor recovery of murine lymphocytes (30). Sucrose dilution procedures such as those used in our rat freezing protocol have been used by Taylor et al. (33) when cryopreserving lymphocytes and macrophages. The same principles of toxicity, permeation and temperature of exposure to DMSO apply to islets but they may not be as sensitive to DMSO. To determine if DMSO toxicity or the osmotic

stresses encountered during the addition or removal of this additive had a deleterious effect on the leukocytes, a series of proper controls should be tested. Fresh islets should be subjected to the entire prefreeze regimen and the sucrose removal of the DMSO. If a difference in xenograft survival between recipients of DMSO-treated fresh islets and cryopreserved islets is also observed, then the effect of the cryopreservation protocol on leukocyte survival can not be attributed to a purely toxic effect of DMSO or to a greater sensitivity to osmotic shock injury. The assumption would then be that, under the particular set of conditions used for islet cryopreservation, the optimal cooling rates for passenger leukocytes and islets differ. Because the critical cryobiological factors are so closely inter-related, they cannot be assessed individually.

Manipulation of various aspects of our standard islet cryopreservation protocol such as DMSO concentration or its extent of permeation would change the optimum cooling rate for islets and may result in a wider gap between optimum rates for leukocytes and islets. Thorpe et al. (30) reported that mouse lymphocytes could handle post-thaw osmotic stress well if they were cooled at their optimal rate (1°C/min). If the cells were cooled at rates above or below the optimum, they were much more sensitive to post-thaw handling procedures such as osmotic stress or centrifugation. Taylor et al. (33) have shown that rat lymphocytes and macrophages cryopreserved with a pre-freeze protocol similar to the islet protocol of Rajotte et al. (5) were destroyed only after cooling at rates greater than 75°C/min. Optimal recovery of these cell types was observed when they were cooled between 0.3 and 5.0°C/min. Viability was assessed by membrane

integrity using a supravital acridine orange and propidium iodide stain. Assessment of viability by dye exclusion has been reported to give much higher viability than other assays (28,30) and does not demonstrate the immunocompetence of the surviving cells. Several studies on leukocyte cryopreservation have indicated that some immunological functions are more susceptible to damage than others even when optimum freeze-thaw conditions are used (28,34,35). Thus, functional viability must be determined by testing for the specific function of interest. Mixed lymphocyte islet cultures (MLIC) in which islets are used as the stimulator population have been used to assess the immunogenicity of isolated islets (36,37). If this assay can be shown to be positively correlated with in vivo transplant results, it could be used to assess passenger leukocyte viability following cryopreservation.

Leukocyte viability can easily be destroyed by cryopreservation protocols if rapid cooling is used, however, it is of utmost importance to ensure maximum islet survival in vivo. Taylor and Benton (38) showed that, after full equilibration in DMSO, islet viability was maintained at cooling rates up to 1000°C/min. Unfortunately the assessment of viability used in this study is questionable. The secretion of insulin during static incubation was calculated by subtracting the post-stimulation nonspecific release from the stimulation level. Several groups have found that cryopreserved islets lose some responsiveness to low glucose and are unable to switch off insulin secretion once glucose stimulation is stopped (7,39,40). In addition, Taylor and Benton cultured islets at 37°C in 95% O_2:5% CO_2 for a minimum of 18 hours prior to freezing. Control islets were

maintained in culture during the freeze, storage and thawing process. Extended periods of culture in 95% / O_2 is likely to damage islets (16) and thus, the observed insensitivity of islets to cooling rate may be a manifestation of dealing with damaged islets. Further studies of islet cryopreservation at more rapid cooling rates are necessary.

It will be of interest to determine which aspects of the cryopreservation protocol alter the immunostimulatory capacity of the intra-islet lymphoid cells. Cryopreservation may destroy passenger leukocytes as tissue culture does or it may simply inactivate the immunocompetency of the cells as does UVB irradiation. Sutton et al. (41) detected class I and class II antigens on human isolated islets before and after cryopreservation. They observed an increase in class I but no change in class II antigen expression. Cells which expressed human leukocyte antigen were similar in fresh, cultured and cryopreserved islets. This suggests that the passenger leukocytes are inactivated rather than depleted by the freeze-thaw process. Thus, although leukocytes may survive, their antigen-presenting capacity may be altered.

CONCLUSIONS AND RECOMMENDATIONS

1. The isologous islet transplantation study has conclusively shown that islets of Langerhans, cryopreserved by slow cooling to -40°C, storage at -196°C and rapid thawing, can reverse experimentally induced diabetes in rats and can do so as well as freshly isolated

2. The standard rodent islet cryopreservation protocol of Rajotte et al. (6) can reduce islet immunogenicity as shown by extended graft survival in a xenograft model.

3. The mechanism by which this cryopreservation protocol reduces islet immunogenicity must be investigated. The effect of DMSO on intra-islet leukocyte viability should be tested in the xenograft model using, as controls, fresh islets which have been exposed to the same pre-freeze and post-thaw regimens as the cryopreserved islets.

4. New cryopreservation protocols should be examined but a more basic approach is suggested. Following various manipulations of the freezing protocol, both islet and leukocyte viability should be tested by reliable in vitro assays. Perifusion and MLIC could be used to assess islet and passenger leukocyte viability, respectively. From these studies an optimal protocol can be chosen for testing in vivo.

5. Successful islet cryopreservation and its effect on xenograft survival are encouraging. Numerous manipulations of the freeze-thaw process must be examined in order to find a protocol which maximizes leukocyte inactivation and maintains islet viability in vivo. Such a protocol would be invaluable to the clinical application of islet transplantation.

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APPENDIX A

ISOGRAFT DATA

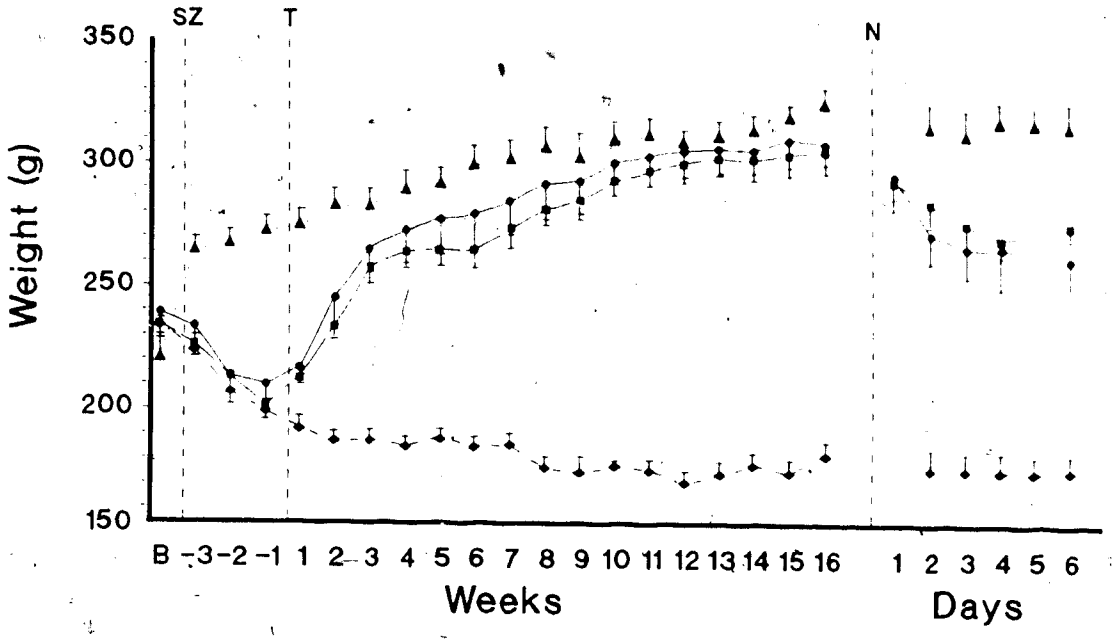


FIGURE A-1. Weight responses of islet isograft recipients and controls. Fresh (n=6 •——•) Cryopreserved (n=4 ▪ - - -▪) Normal controls (n=4 ▲ ▲) Diabetic controls (n=4 ◆ ◆) B - baseline, SZ - streptozotocin, T - transplantation, N - nephrectomy.

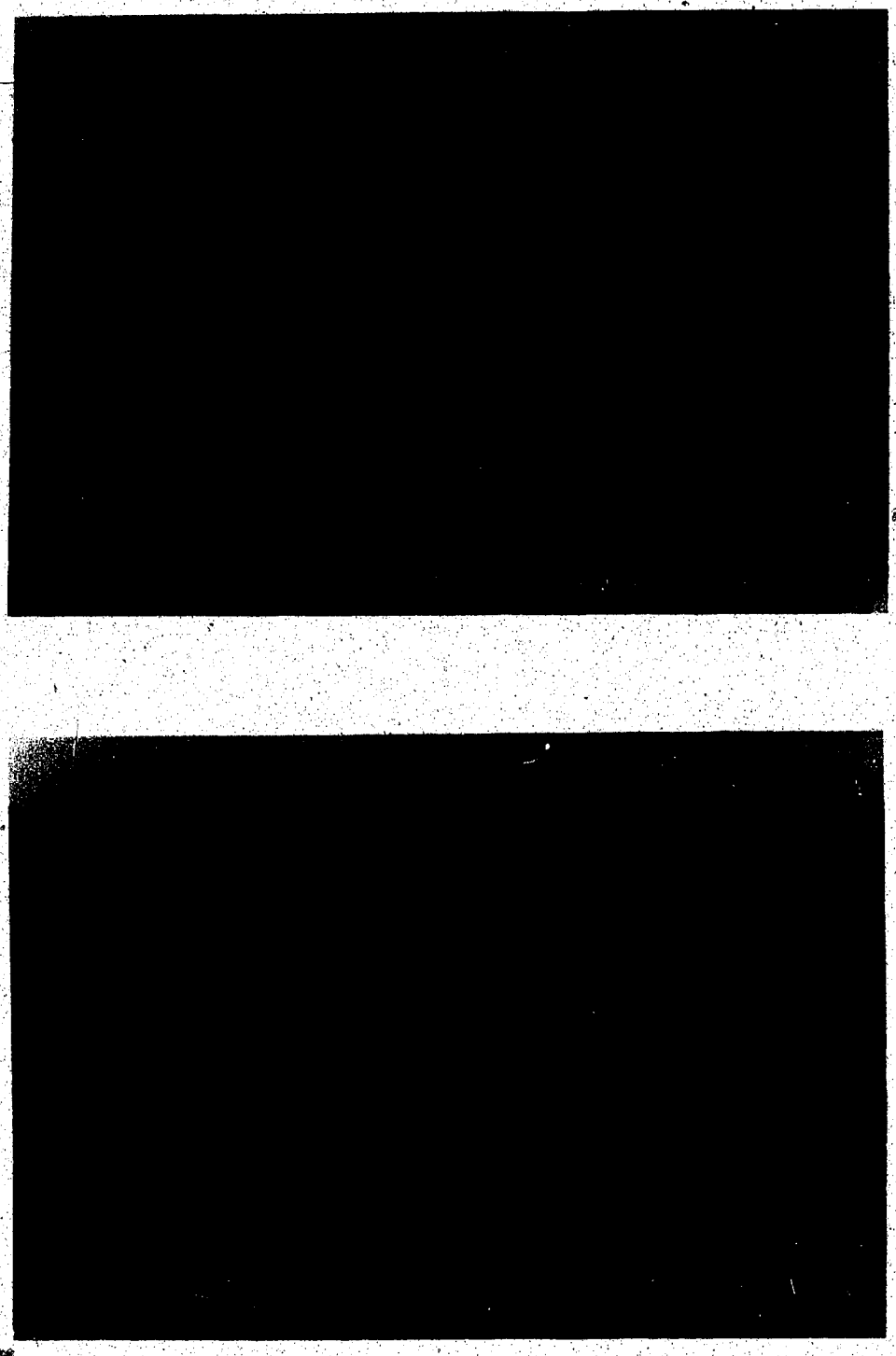


PLATE A-1. Rat islet isografts beneath the kidney capsule four months after transplantation.
(A) Fresh. **(B)** Cryopreserved. Aldehyde fuchsin stain.

APPENDIX B

ALLOGRAFT DATA

Donors: Wistar-Furth rats ($RT1^u$)

Recipients: Lewis rats ($RT1^l$)

Streptozotocin: 60 mg/kg intravenously

Transplantation: 3000 handpicked clean islets were implanted beneath the kidney capsule

Cyclosporin-A dose: 30 mg/kg given subcutaneously on days -1, 0, +1, and +2 with respect to transplant day 0.

Nephrectomy: Graft-bearing kidneys were removed two weeks after transplantation in groups 1 and 2 and at six weeks post-transplant in groups 3 - 6.

GROUPS	TREATMENT	n
1	FRESH ISLETS	6
2	CRYOPRESERVED ISLETS	6
3	FRESH + CyA	8
4	CRYO + CyA	10
5	NORMAL CONTROLS	6
6	DIABETIC CONTROLS	6

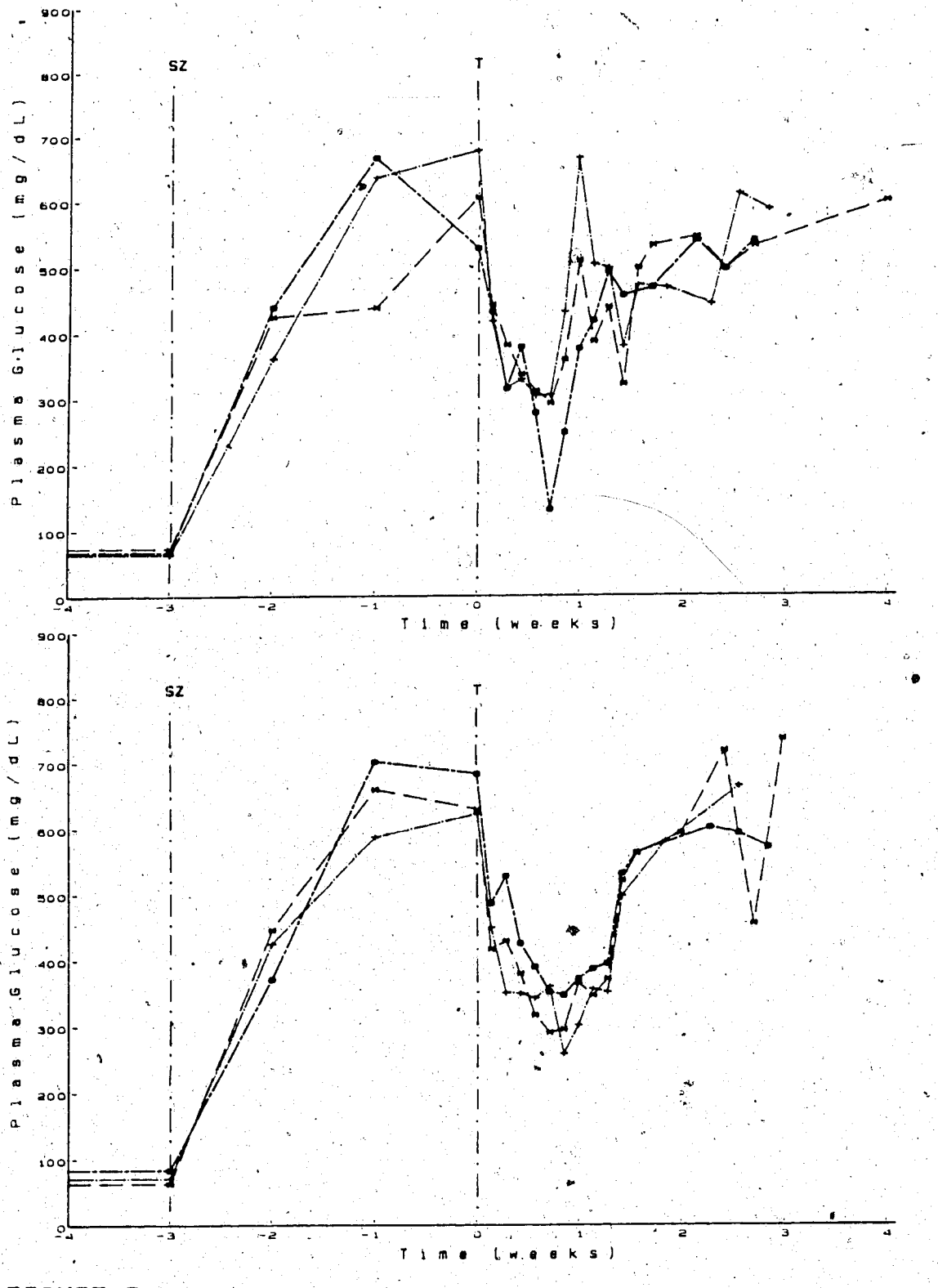


FIGURE B-1. Individual plasma glucose responses of fresh islet allograft recipients (n=6). Nephrectomies performed two weeks post-transplant.
SZ - streptozotocin, **T** - transplantation.

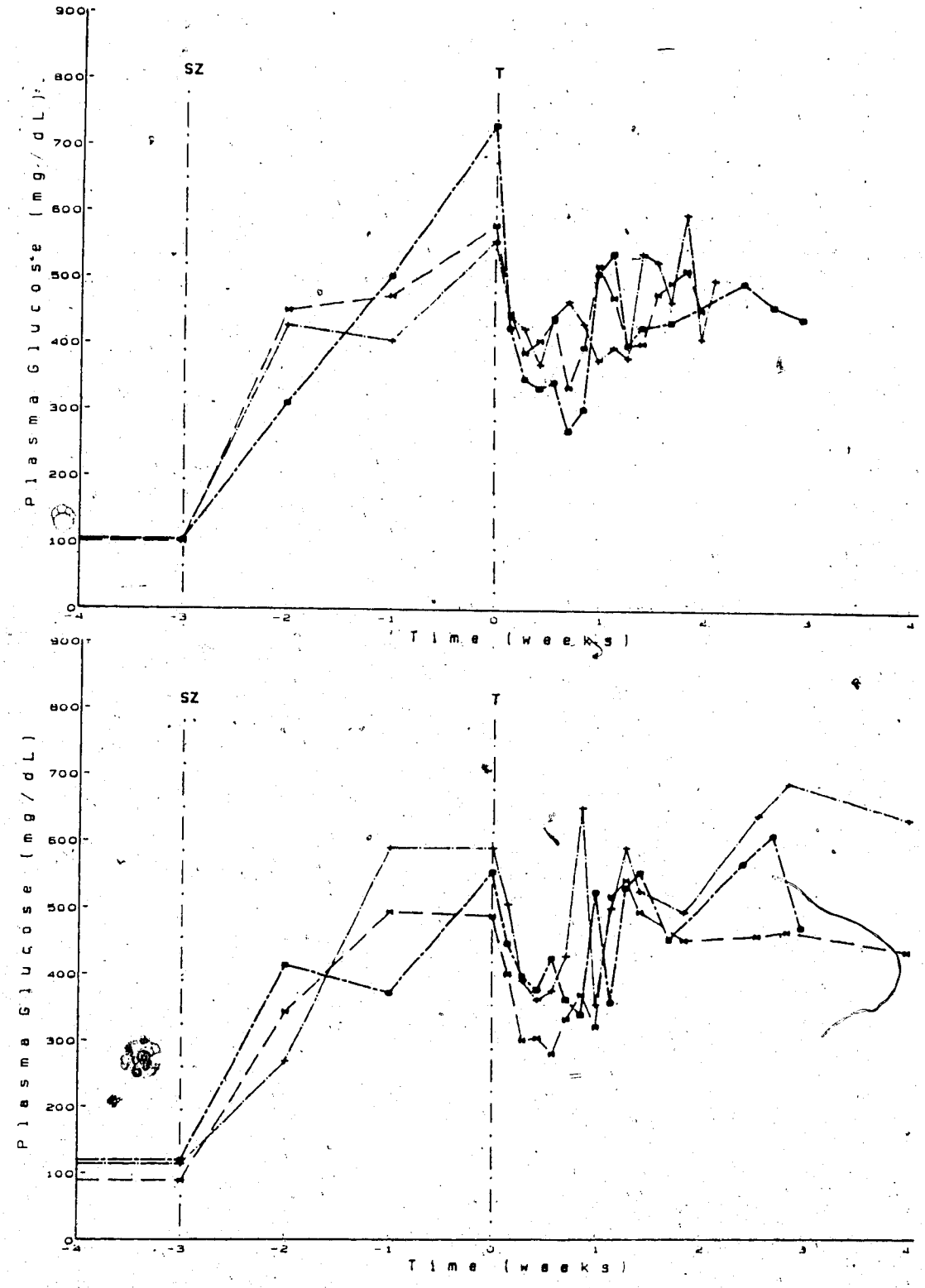


FIGURE B-2. Individual plasma glucose responses of cryopreserved islet allograft recipients (n=6). Nephrectomies performed two weeks post-transplant. **SZ** - streptozotocin, **T** - transplantation.

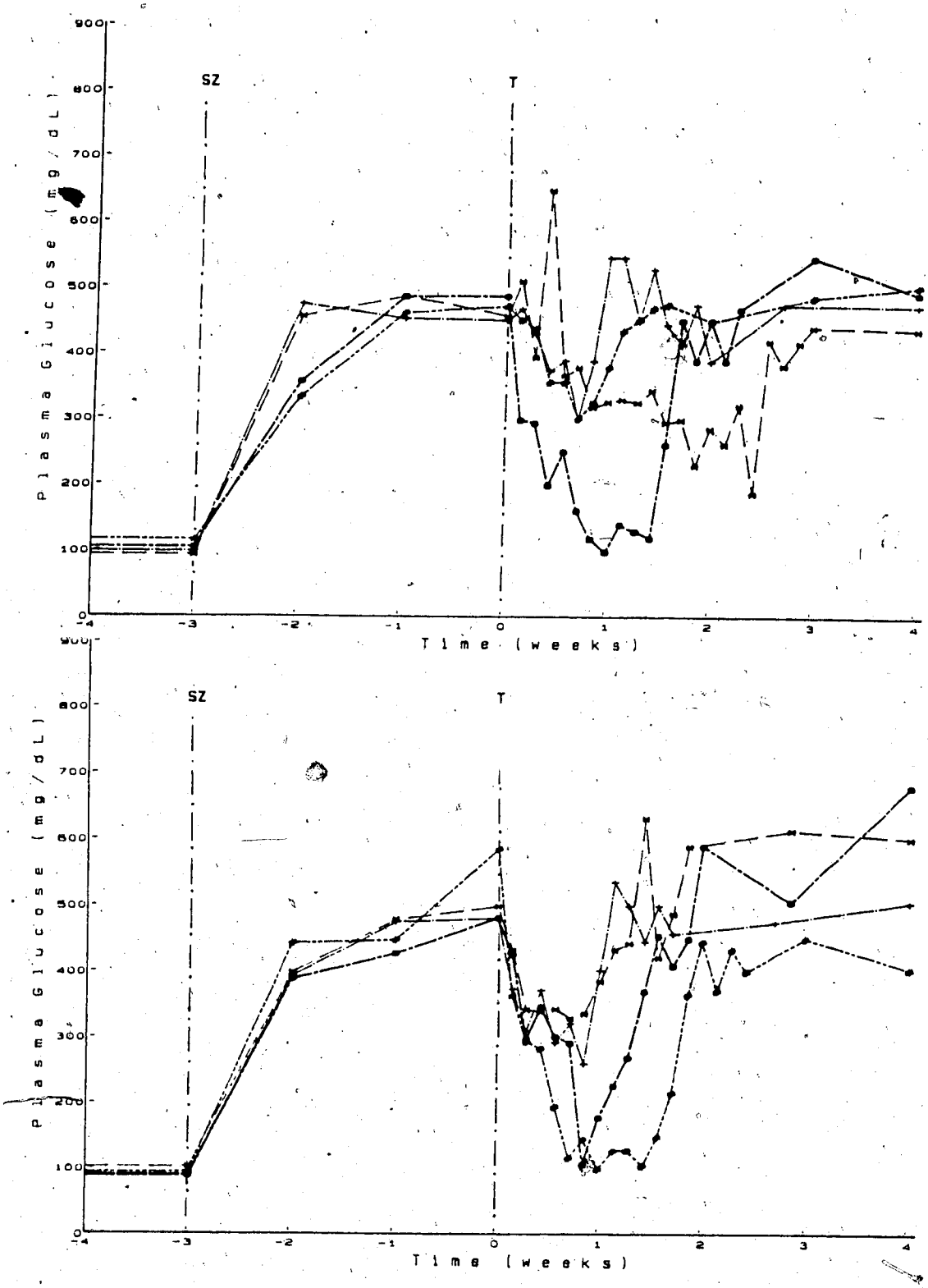


FIGURE B-3. Individual plasma glucose responses of fresh islet allograft recipients given CyA (n=8).
SZ - streptozotocin, **T** - transplantation

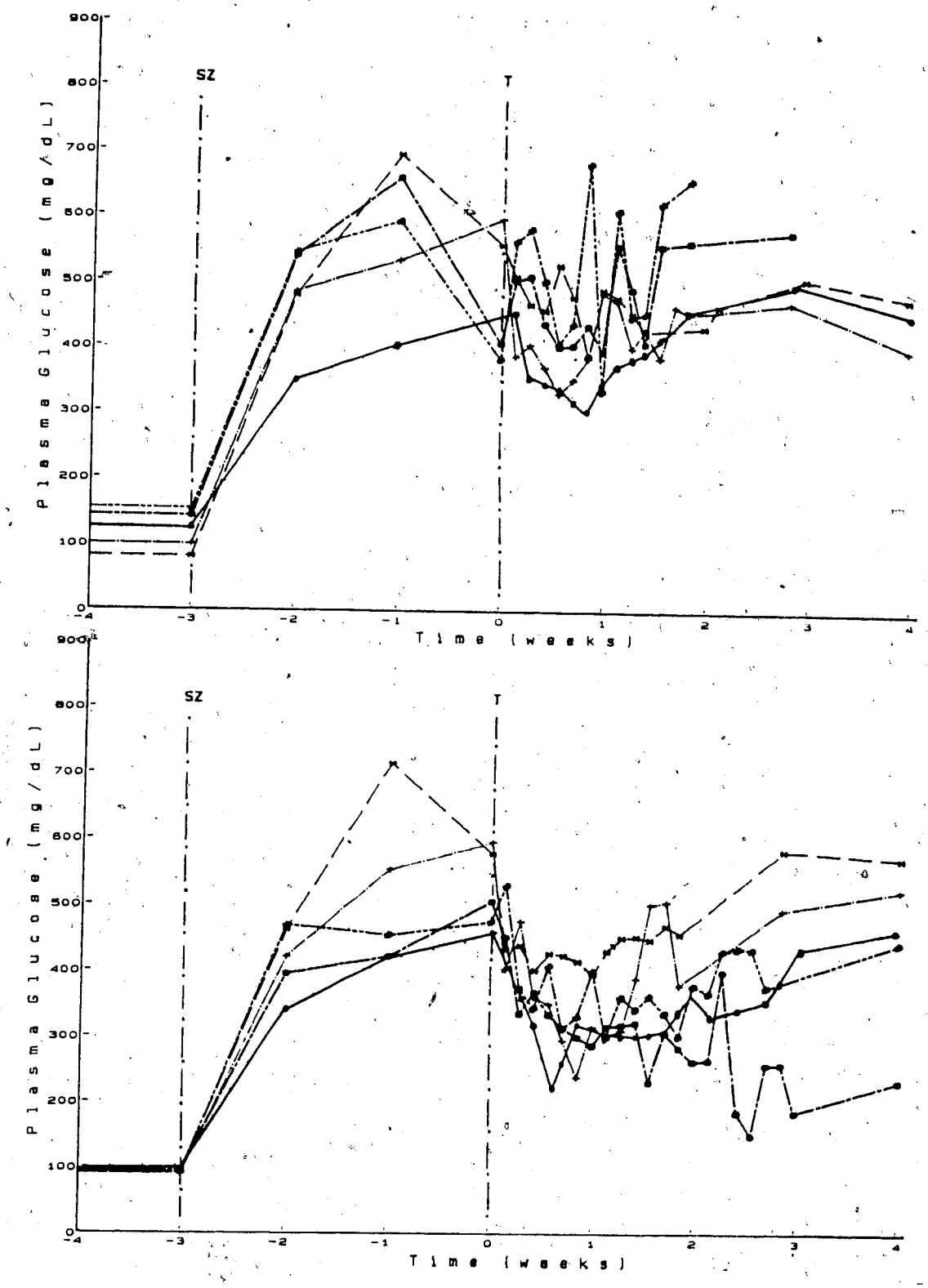


FIGURE B-4. Individual plasma glucose responses of cryopreserved islet allograft recipients given CyA (n=10). **SZ** - streptozotocin, **T** - transplantation

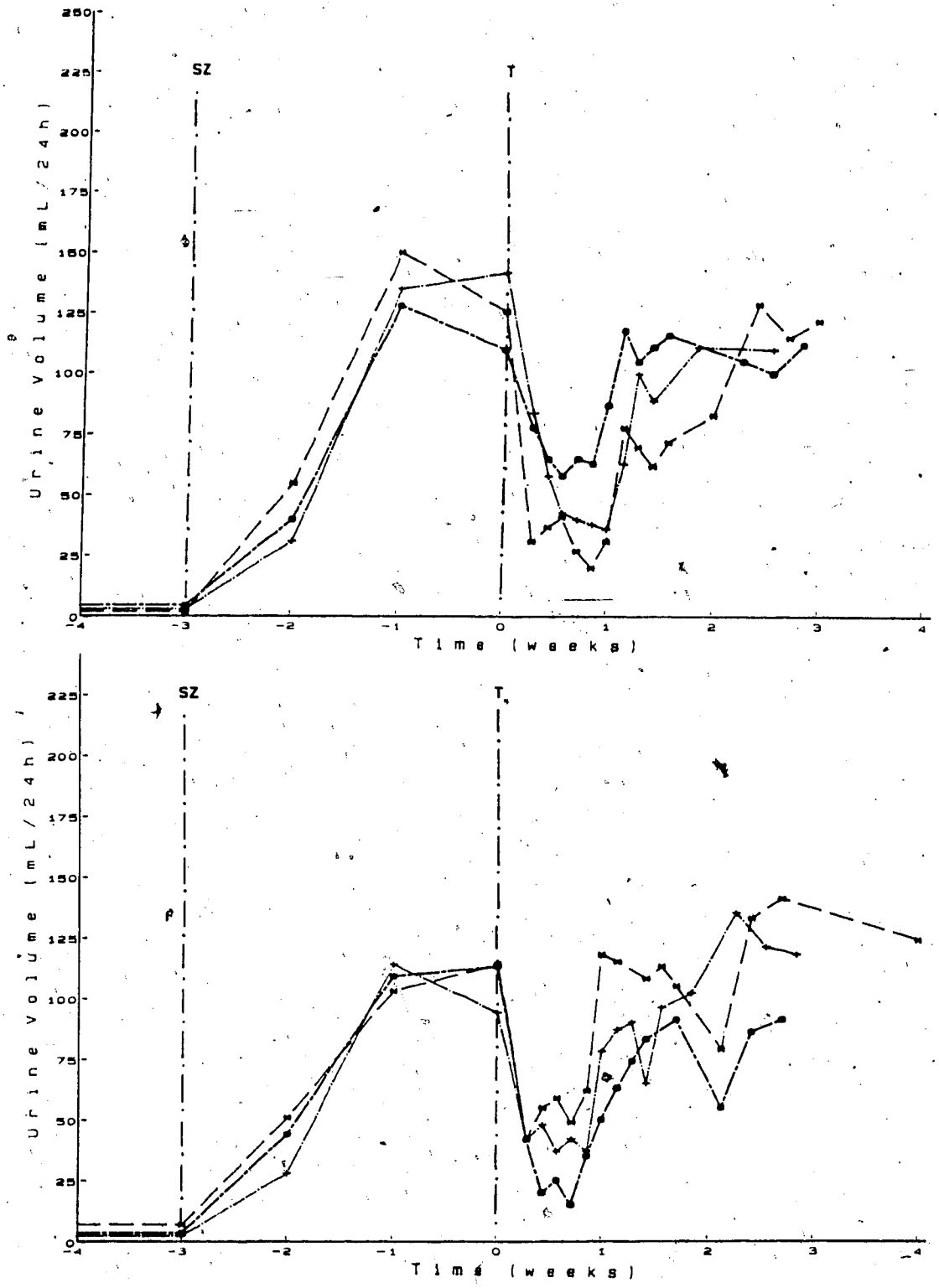


FIGURE 8-5. Individual urine volume responses of fresh islet allograft recipients (n=6). Nephrectomies performed two weeks after transplantation.
SZ - streptozotocin, **T** - transplantation.

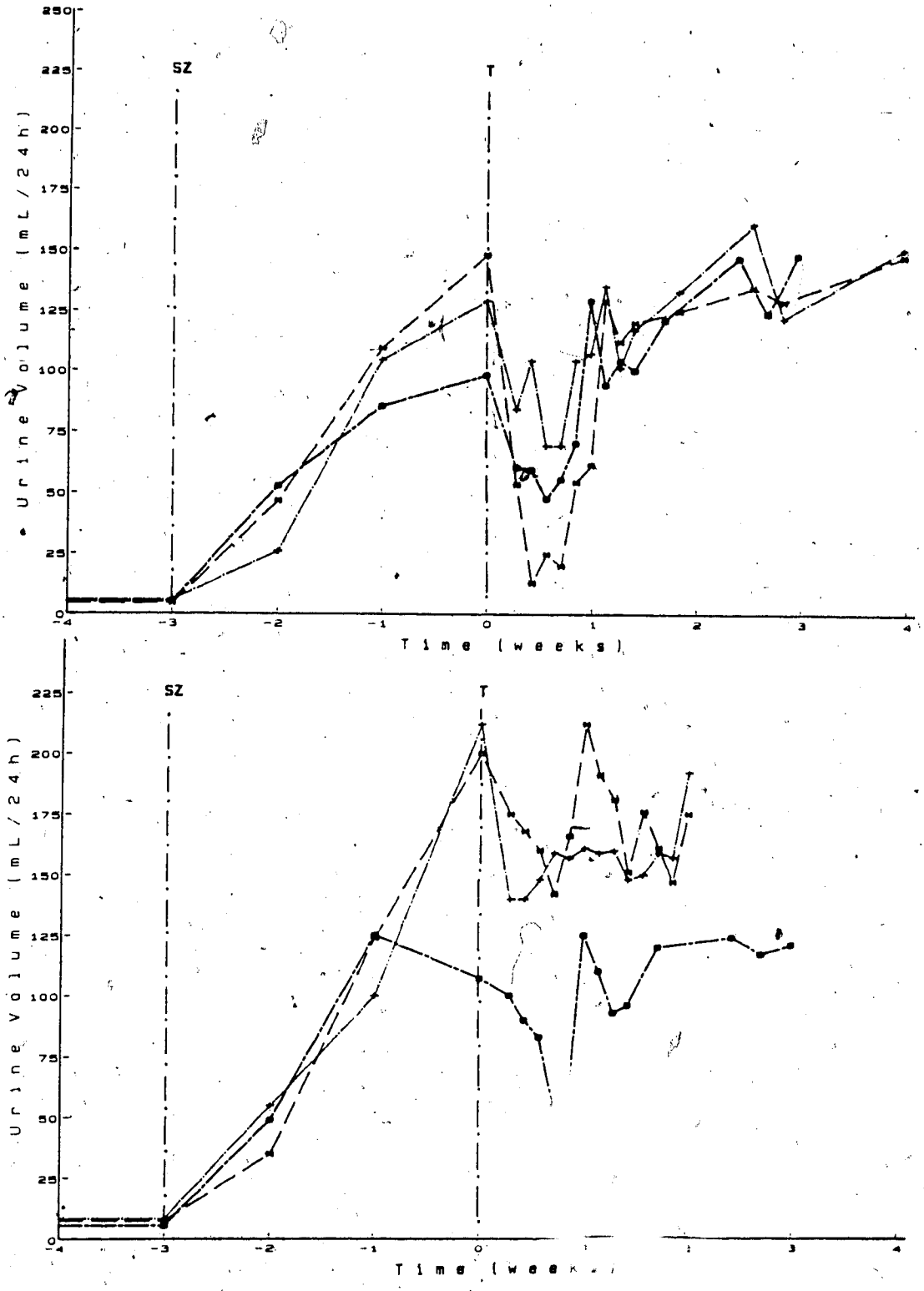


FIGURE B-6. Individual urine volume responses of cryopreserved islet allograft recipients (n=6). Nephrectomies performed two weeks after transplantation. **SZ** - streptozotocin, **T** - transplantation.

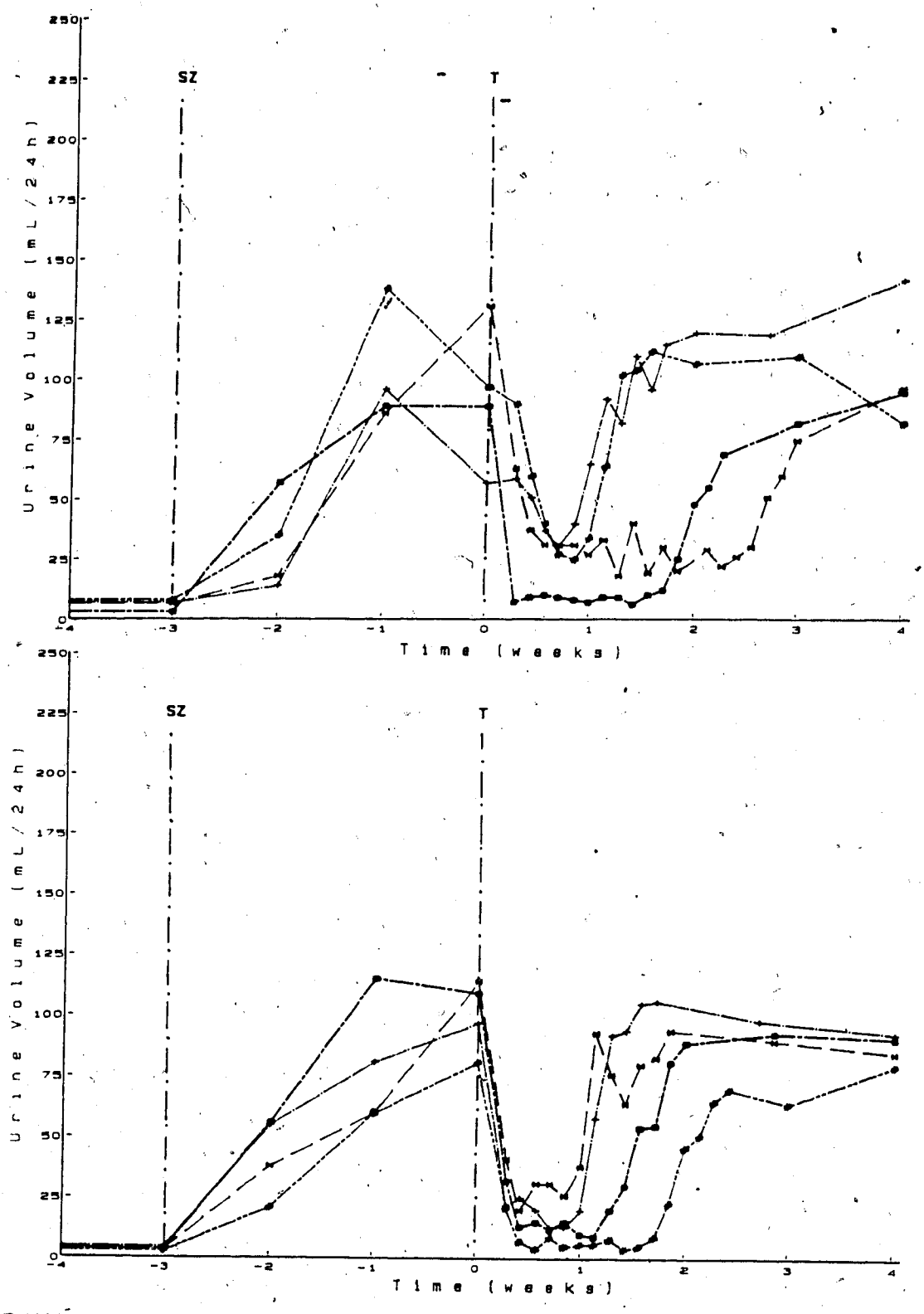


FIGURE B-7. Individual urine volume responses of fresh islet allograft recipients given CyA (n=8). **SZ** - streptozotocin, **T** - transplantation.

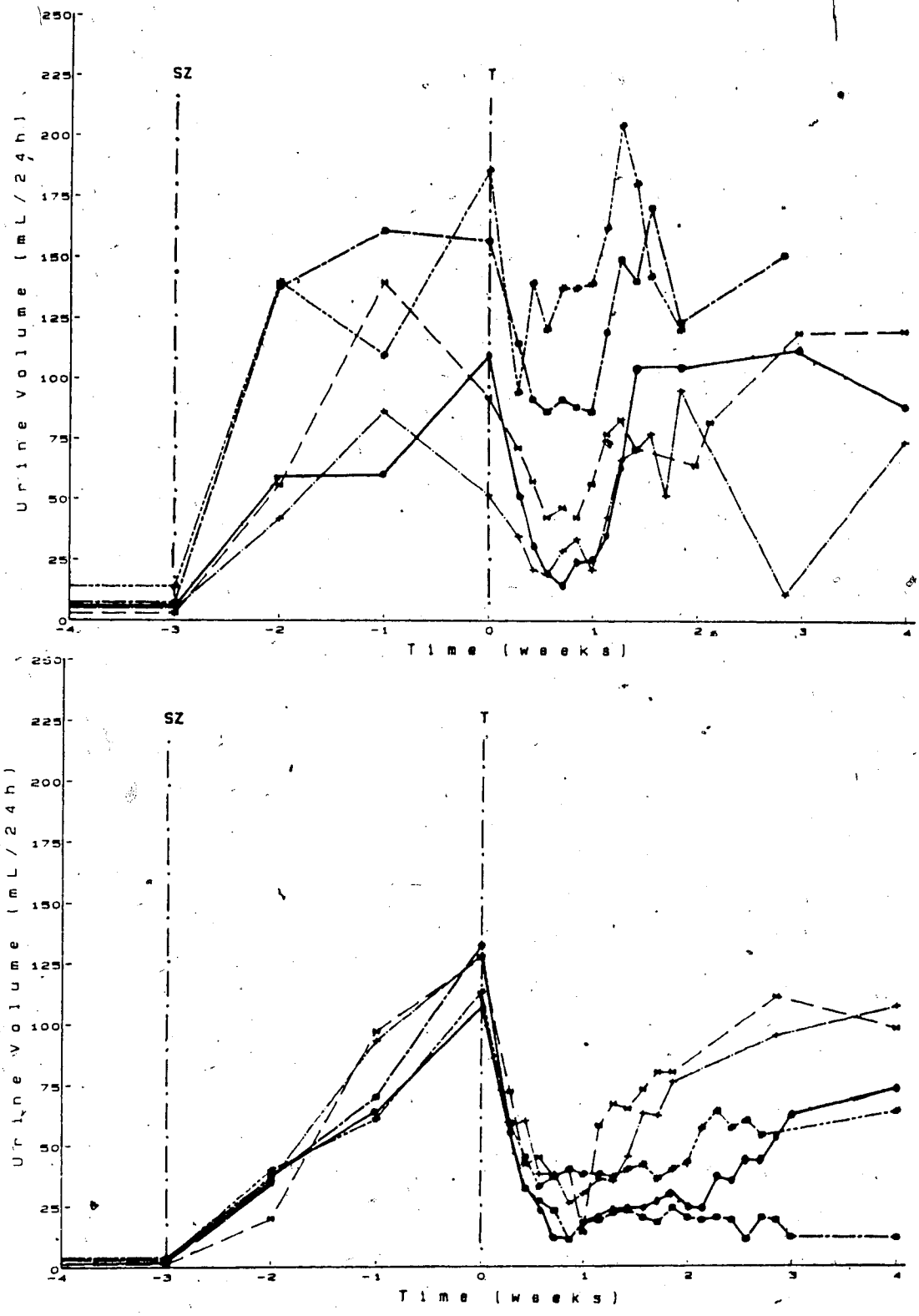


FIGURE 8-8. Individual urine volume responses of cryopreserved islet allograft recipients given CyA. **SZ** - streptozotocin, **T** - transplantation.

APPENDIX C

XENOGRAFT DATA

Islet donors: Wistar-Furth rats

Recipients: BALB/c mice

Transplant site: kidney capsule.

TABLE C-1 Effect of islet number and anti-lymphocyte serum on the success of islet transplantation in a xenograft model.

Number of islets	Number of transplants	Deaths post-transplant	Technical failures	Successful implants
900	3 Fresh	3	---	---
700	7 Fresh	3	---	4
	6 Cryo	2	---	4
500	11 Fresh	---	---	11
	13 Cryo	3	2	8
	22 Fresh + ALS	6	---	16
	19 Cryo + ALS	6	2	11

GROUP	TREATMENT	BASELINE	ONE WEEK PRE-TRANSPLANT	ONE WEEK POST-TRANSPLANT
1	FRESH	133.4± 3.1	644.4±18.4	84.6± 4.0
2	CRYO	136.6± 5.0	666.6±17.4	95.1± 7.3
3	FRESH + ALS	138.6± 4.7	631.0±18.6	104.9± 7.3
4	CRYO + ALS	145.8± 4.3	593.6±37.1	109.4±15.2

TABLE C-2. Plasma glucose concentrations (mean ± SE, mg/dL) of streptozotocin-induced diabetic mice transplanted with 500 fresh or cryopreserved rat islets, with or without anti-lymphocyte serum (ALS).

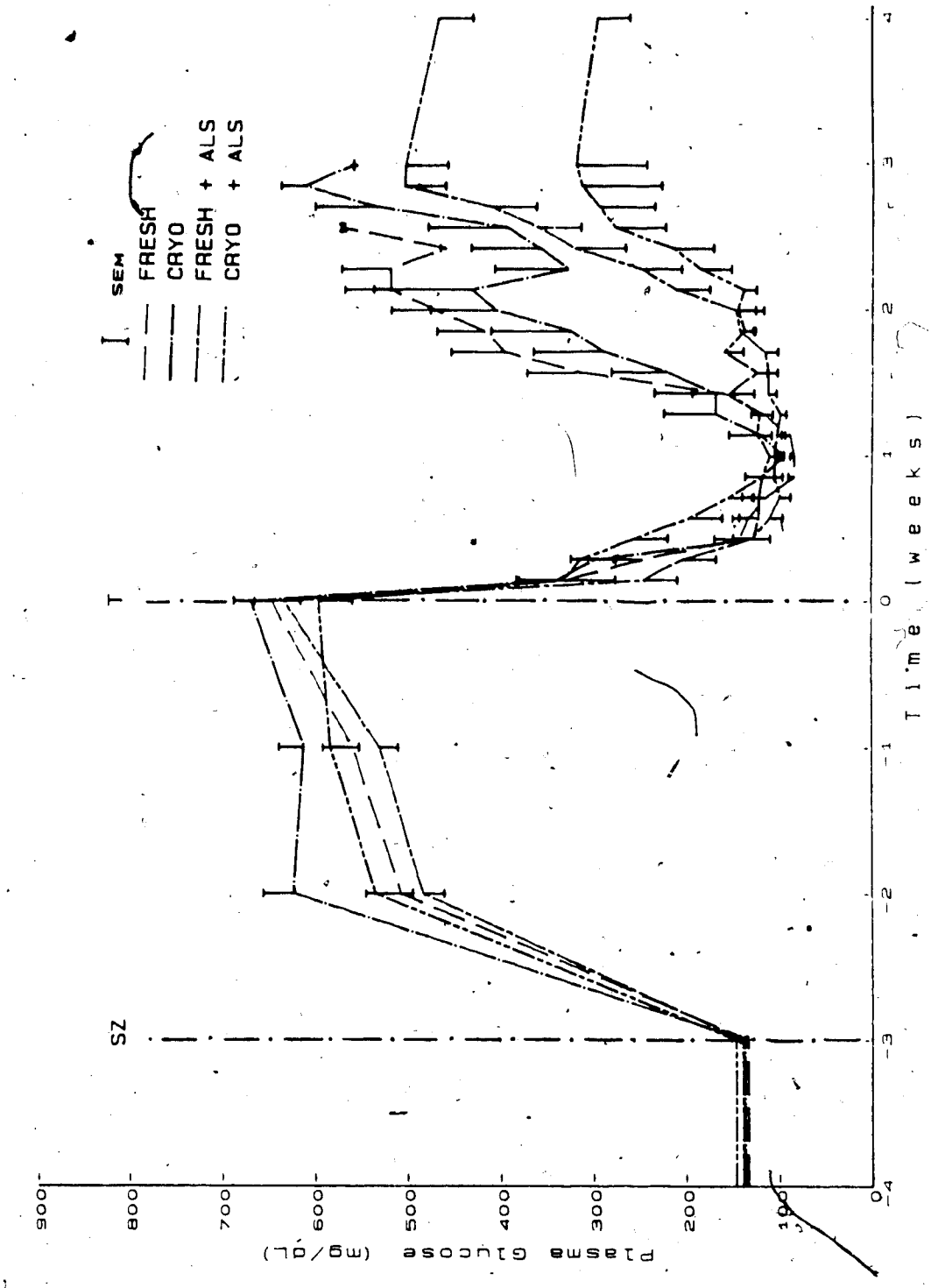


FIGURE C-1. Plasma glucose responses of islet xenograft recipients. **SZ** - streptozotocin, **T** - transplantation

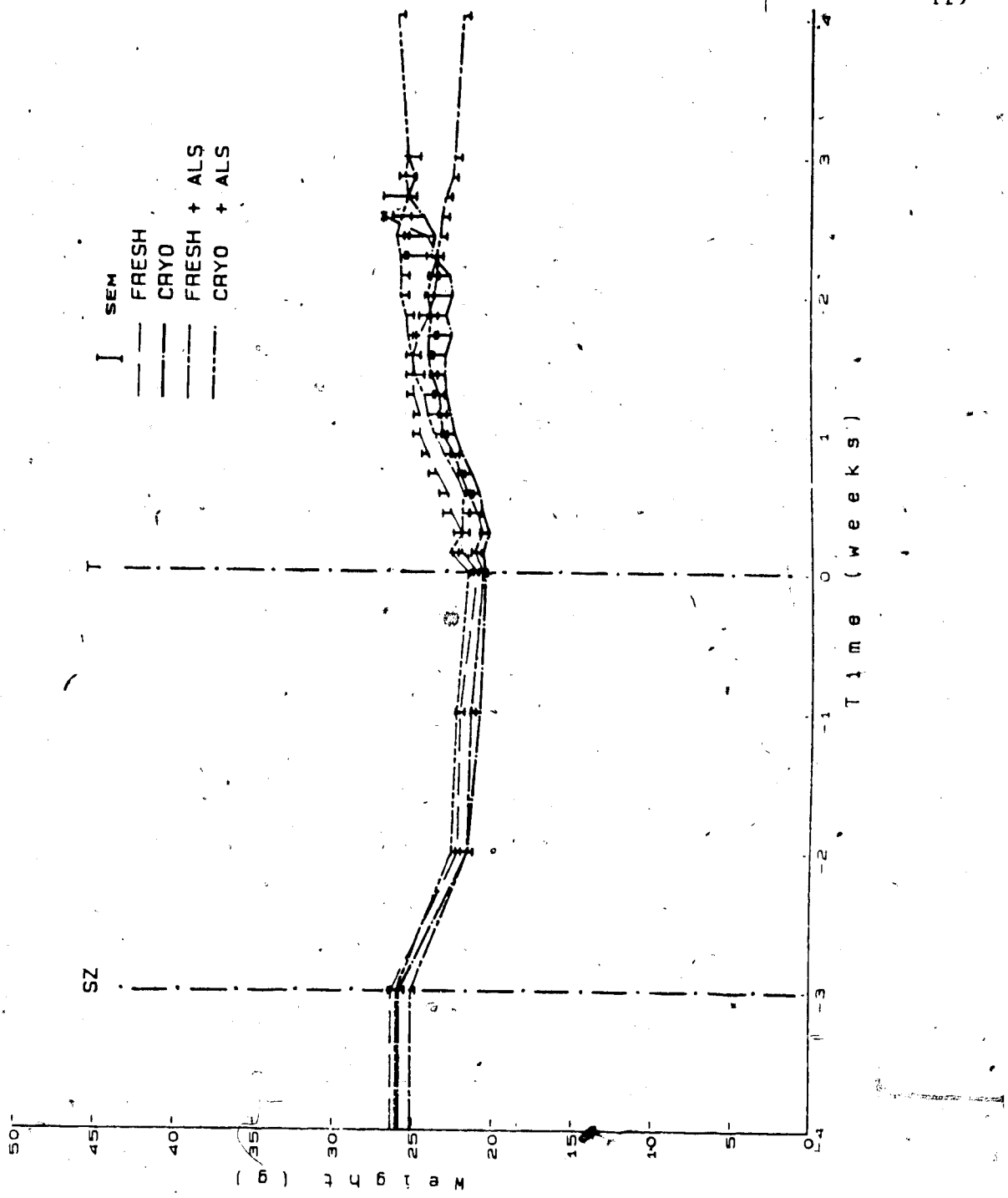


FIGURE C-2. Weight responses of islet xenograft recipients. **SZ** - streptozotocin, **T** - transplantation.

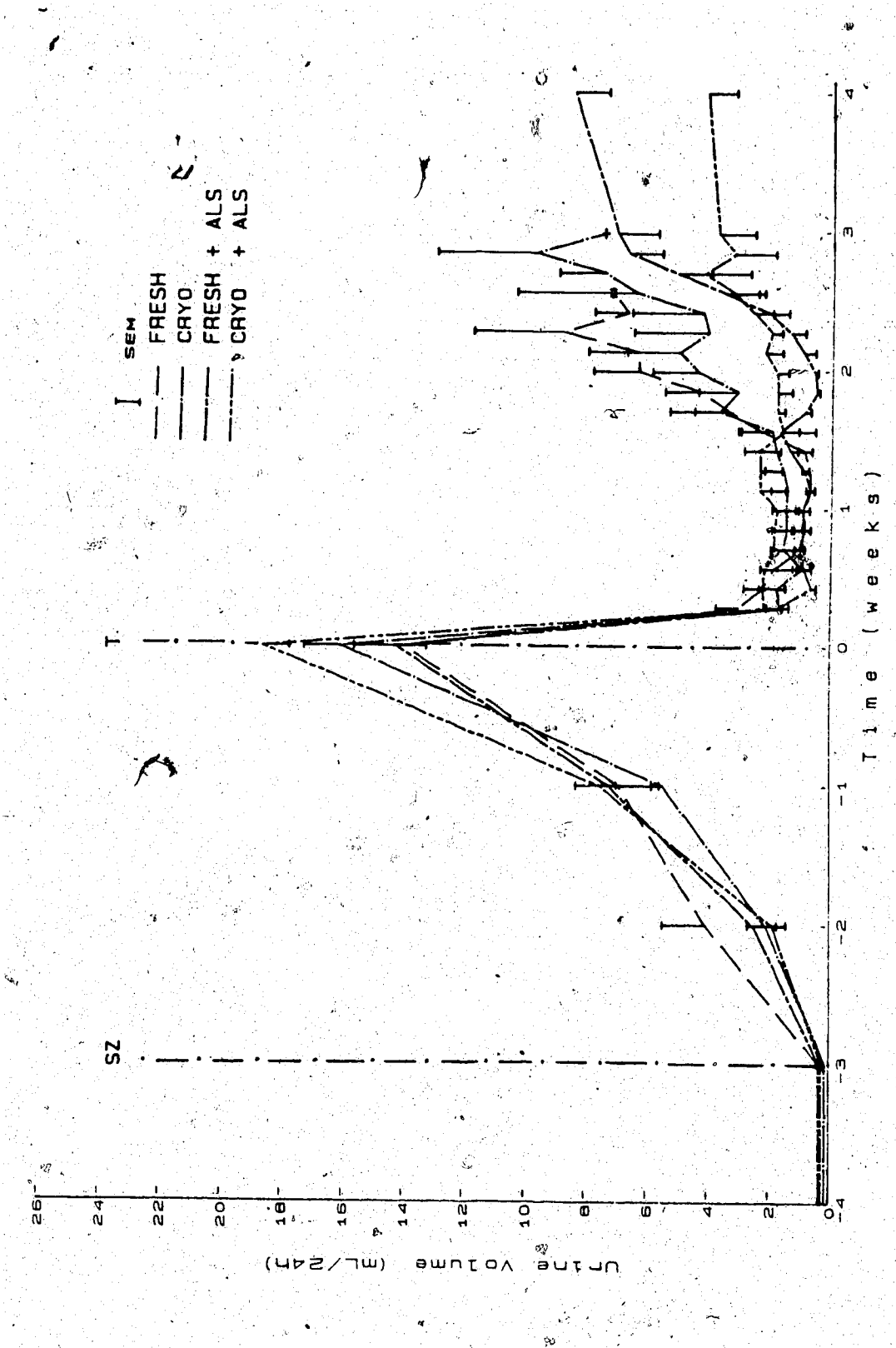


FIGURE C-3. Urine volume responses of islet xenograft recipients. **SZ** - streptozotocin, **T** - transplantation.

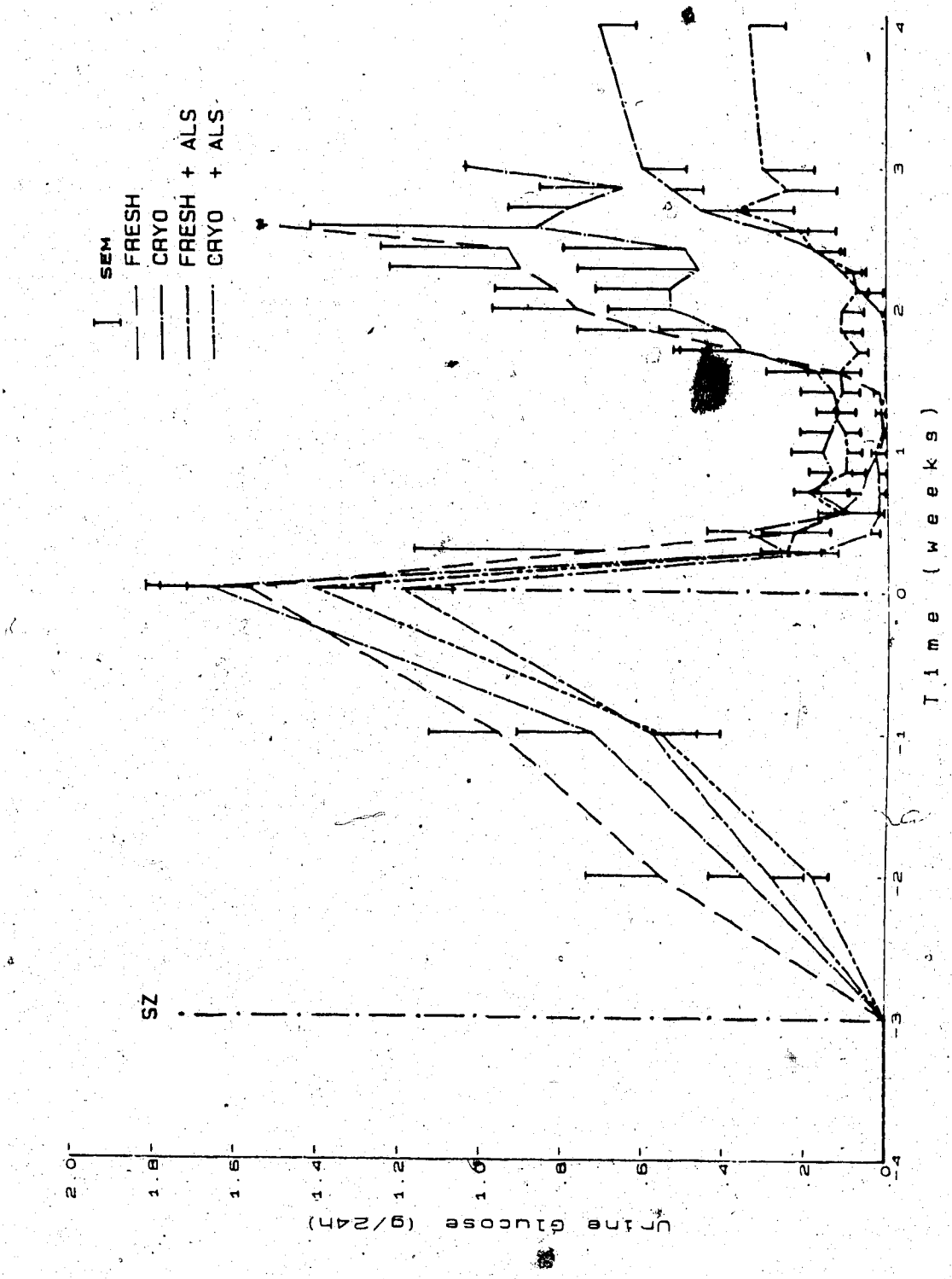


FIGURE C-4. Urine glucose responses of islet xenograft recipients. **SZ** - streptozotocin, **T** - transplantation.

BALB/c CONTROLS

Diabetic controls (DC): received 220 mg/kg SZ

Normal controls (NC): received acetate buffer instead of SZ

NC: n=12 NC + ALS: n=6

DC: n=15 DC + ALS: n=7

Controls received sham transplants two weeks after injection of SZ or acetate buffer. Five diabetic controls died prior to the sham-transplant.

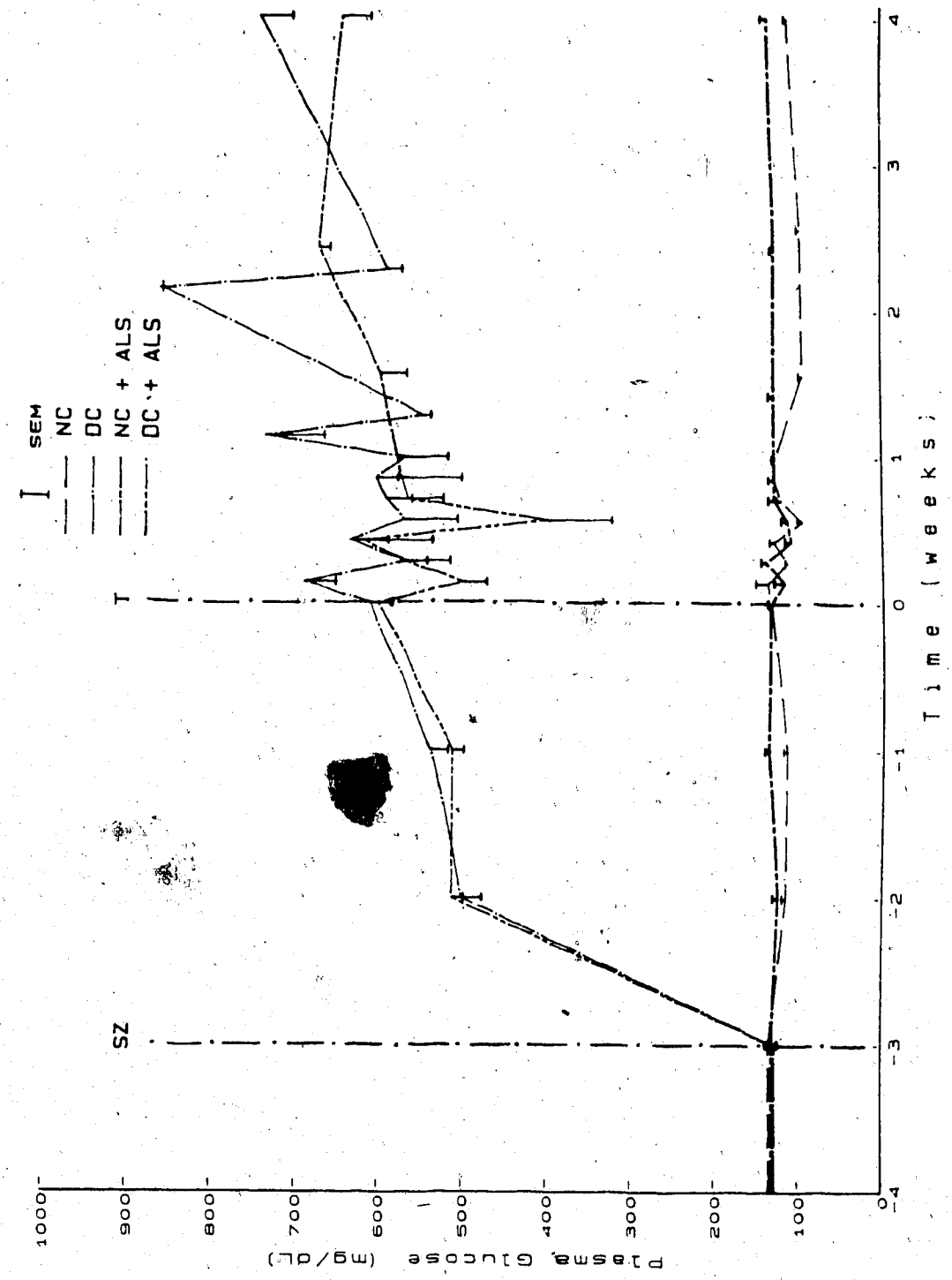


FIGURE C-5. Plasma glucose responses of normal **(NC)** and diabetic **(DC)** BALB/c controls. **SZ** - streptozotocin or acetate buffer. **T** - sham-transplant.

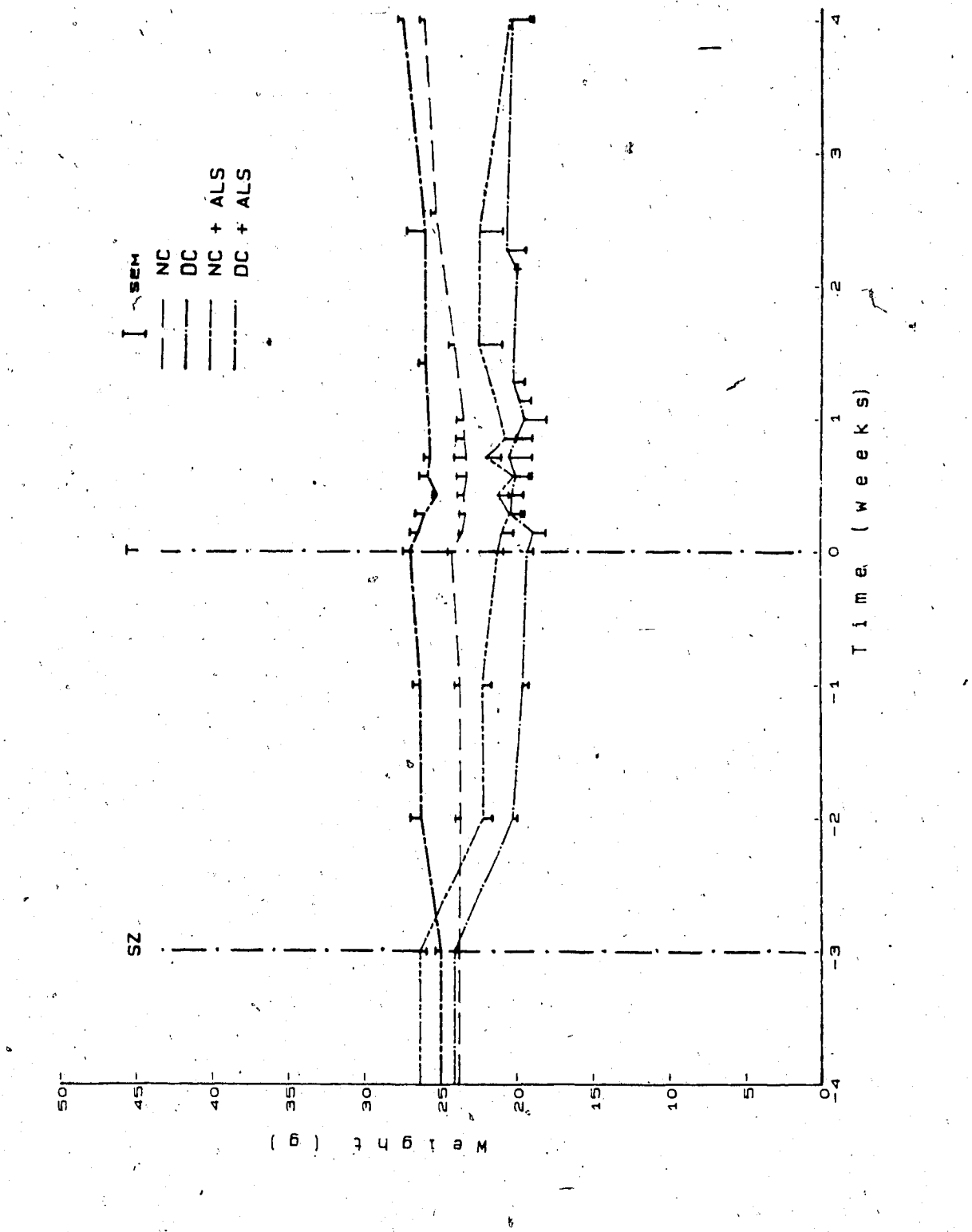


FIGURE C-6. Weight responses of normal **(NC)** and diabetic **(DC)** BALB/c controls. **SZ** - streptozotocin or acetate buffer. **T** - sham-transplant.

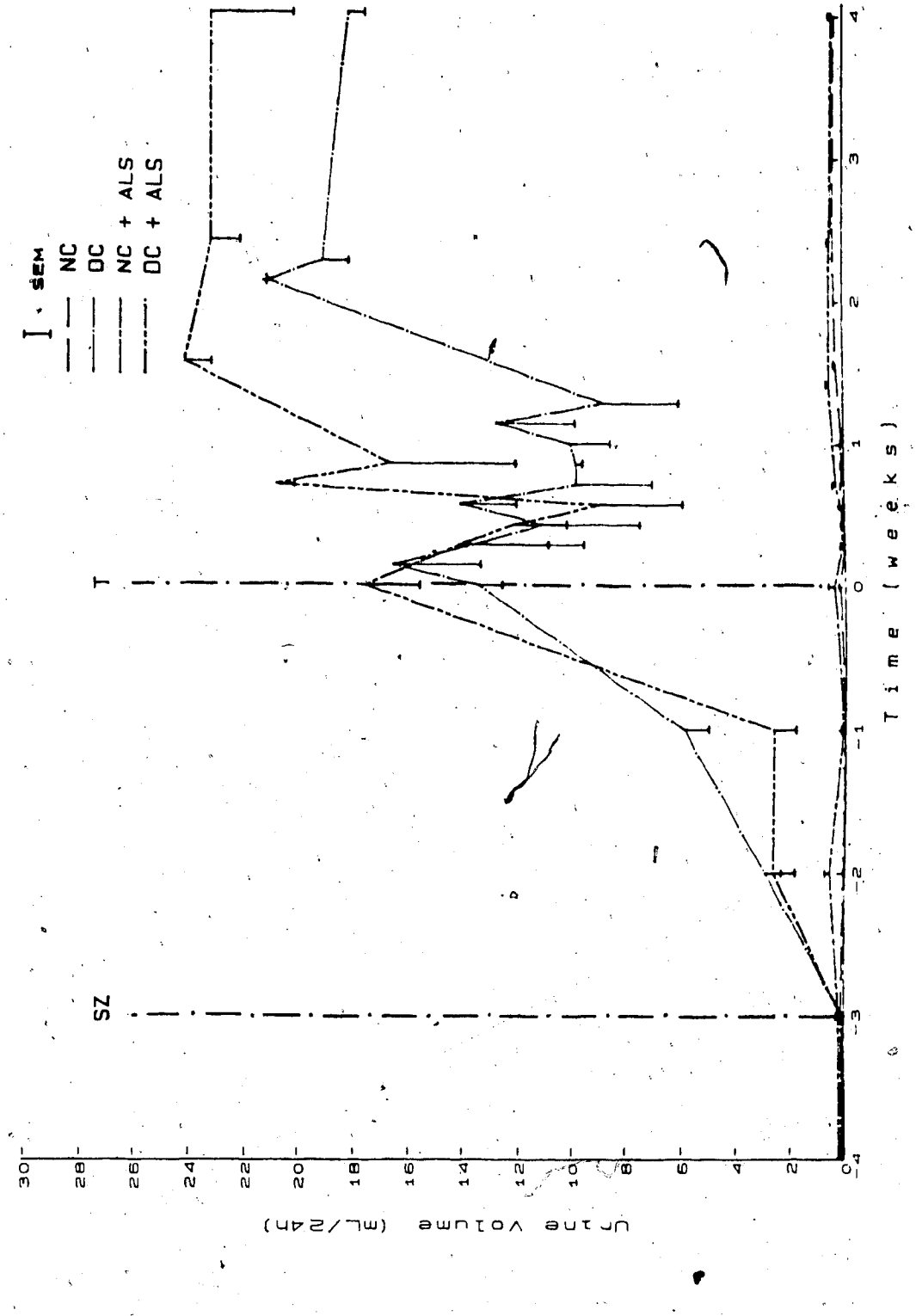


FIGURE C-7. Urine volume responses of normal **(NC)** and diabetic **(DC)** BALB/c controls. **SZ** - streptozotocin or acetate buffer. **T** - sham-transplant.

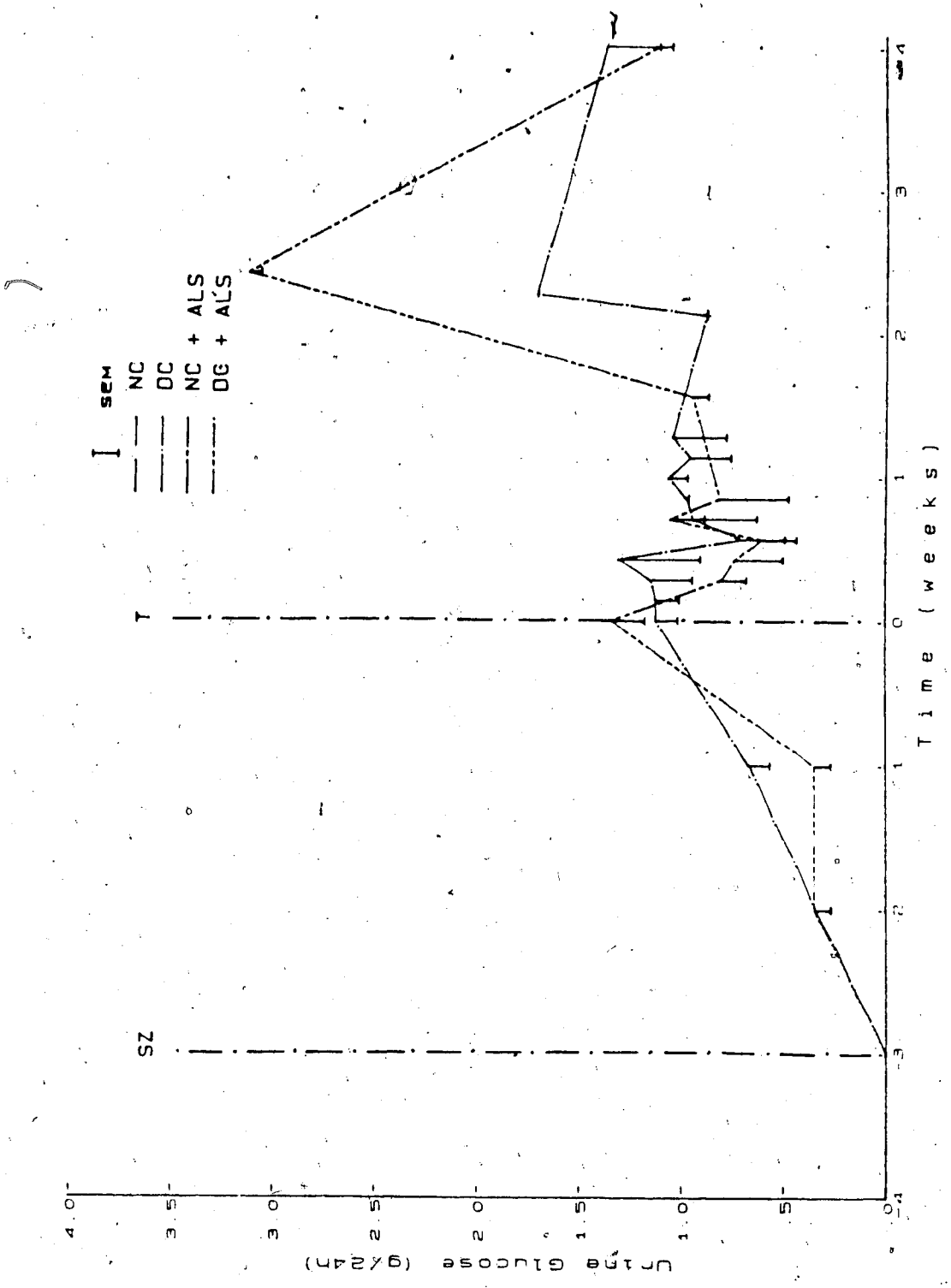


FIGURE C-8. Urine glucose responses of normal **(NC)** and diabetic **(DC)** BALB/c controls. Normal control urine glucose is < 0.0010 g/24h. **SZ** - streptozotocin or acetate buffer. **T** - sham-transplant.

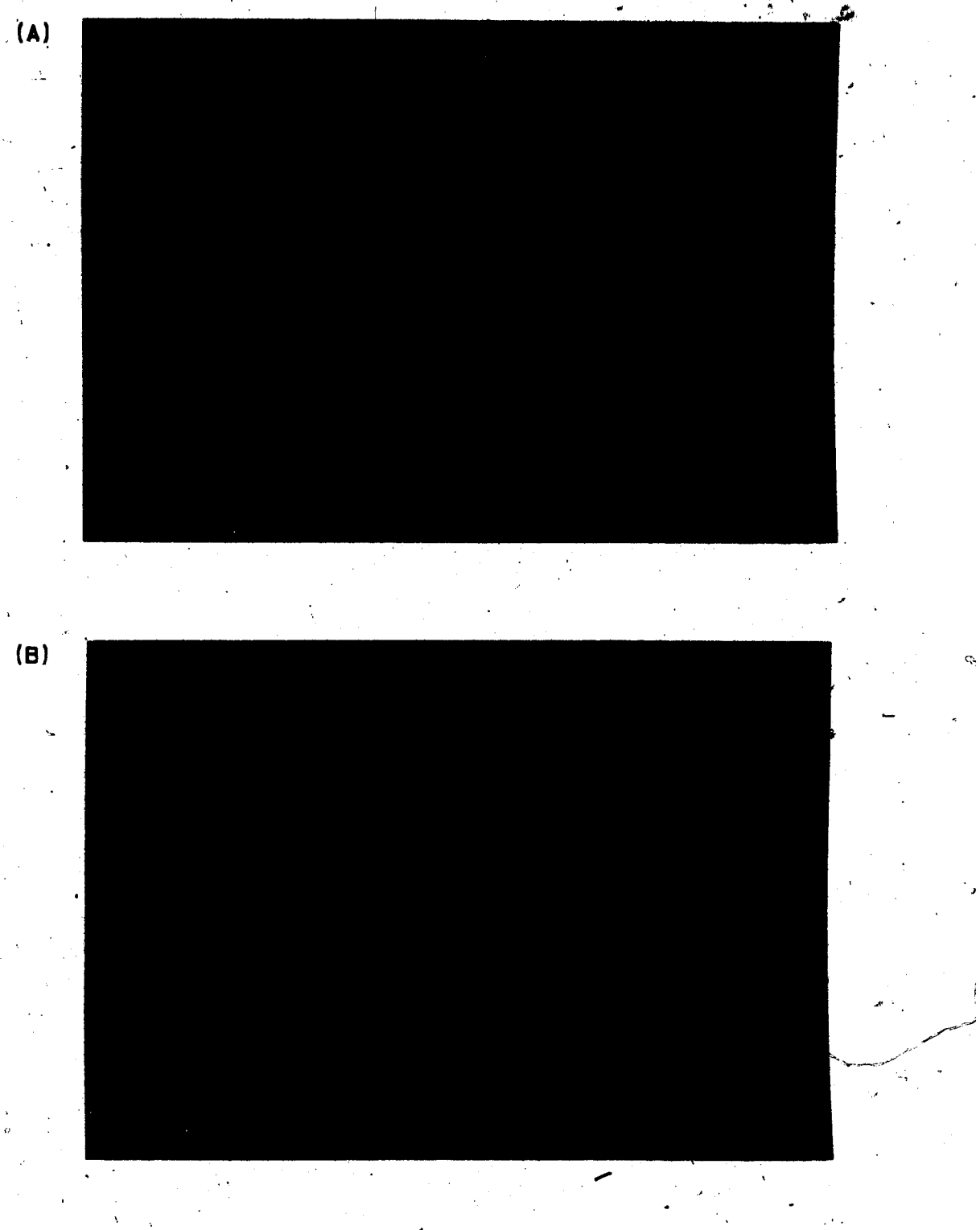


PLATE C-1. Freshly isolated rat islets implanted beneath the kidney capsule of diabetic mice. **(A)** Four days post-transplant. **(B)** Extensive lymphocytic infiltration at six days post-transplant. Immunoperoxidase stain.

APPENDIX D

METHODS AND MATERIALS

EXPERIMENTAL ANIMALS

(i) Rats: males 200-300g inbred Wistar-Furth ($RT1^u$), donors
males 200-300g inbred Lewis ($RT1^l$), recipients

(ii) Mice: males 25-30g inbred BALB/c ($H\text{-}2^d$), recipients

INDUCTION OF DIABETES

Mice were anesthetized with Avertin (1); tribromoethanol in amyl alcohol (0.015 mL/g body wt). Rats were lightly anesthetized with ether. Diabetes was induced in mice by an injection of streptozotocin (SZ) (Upjohn, Kalamazoo, Michigan) (220 mg/kg body weight) via the retroorbital sinus and in rats (60 mg/kg body weight) via the penile vein. SZ was dissolved immediately before use in 0.1 M acetate buffer (pH 4.5) made up in 0.9% saline.

ISLET ISOLATION

Six inbred WF rats were anesthetized with sodium pentobarbitol (40 mg/kg) (Somnotol, MTC Pharmaceuticals, Hamilton, Canada). The pancreases were cannulated via the common bile duct with Intramedic non-radiopaque PE-50 polyethylene tubing (Clay Adams, B-D Co., Parsippany, NJ) and distended with 10 mL of chilled Hanks Balanced Salt Solution (HBSS, Gibco, Grand Island, NY) containing 2.0 mg/mL dextrose (Abbott Laboratories,Ltd.),penicillin (225 U/mL) and streptomycin (225 μg/mL) (Whittaker M.A. Bioproducts, Walkersville, MD). The pancreases were rapidly excised and placed in chilled HBSS. When all pancreases

were removed, they were picked free of contaminating lymph nodes and fatty tissue, placed in chilled HBSS and then chopped vigorously with scissors. The minced tissue was divided equally into two preweighed Falcon conical centrifuge tubes and spun at 450g. The supernate was carefully suctioned off and the tubes were weighed. HBSS (2.0 mL/g tissue) and 15 mg/g tissue Type V collagenase (Sigma, St. Louis, MO.) were added immediately before clamping to a Gallenkamp flask shaker in a 37°C water bath. The minced tissue was digested for 8 minutes at 8 cycles/sec and then shaken by hand until an individually assessed digestion endpoint was reached. Digestion was stopped by the addition of chilled HBSS to a volume of 30 mL. The tubes were spun at 450g and the supernate aspirated. Any remaining collagenase and exocrine debris were removed by a series of washes using 15 mL HBSS and centrifugations at 450, 200, 120, 50 and 450 g. Following the final wash, the supernate was aspirated, 5 mL of 25% Ficoll were added to the tissue and the tubes were lighcly vortexed. Three mL of each of 23%, 20% and 11% Ficoll solutions were carefully layered on top. The gradients were centrifuged at 800g for 20 minutes. Isolated islets of Langerhans, found at the interface of the 23% and 20% Ficoll layers, were removed with a siliconized Pasteur pipette and placed in siliconized Pyrex test tubes. The islets were washed 3 times with Medium 199 containing 25 mM Hepes Buffer, Earle's Salts and L-Glutamine (Gibco, Grand Island, NY), and supplemented with 10% fetal bovine serum (Gibco), penicillin (100 U/mL) and streptomycin (100 μg/mL). The islets were transferred to a petri dish containing supplemented Medium 199 and were viewed under a Wild Leitz dissecting microscope (250X) using a modification of the reflected green light technique of Finke et al. (2). This initial

islet preparation is shown in Plate D-1 (A). Islets, 100 μm - 500 μm and free of any exocrine debris, were handpicked with a finely drawn siliconized Pasteur pipette and counted (Plate D-1 (B)).

CRYOPRESERVATION PROTOCOL

FREEZING PROCEDURE (FIGURE D-1).

To minimize osmotic stress which may result from a hyperosmotic cryoprotectant, DMSO was added in a stepwise fashion to islets, resuspended in 0.2 mL of supplemented Medium 199. At 25°C, 0.1 mL of 2 M DMSO was added for 5 min, followed by a second addition of 0.1 mL of 2 M DMSO for 25 min. To reach a final concentration of 2 M DMSO, the islets were equilibrated in 0.4 mL of 3 M DMSO for 15 min at 0°C. The samples were then transferred to a -7.3°C ethanol seeding bath for 5 min at which time the islets were gently resuspended and nucleation was induced by touching the side of the tube with a cold metal rod. Ten minutes were allowed for the release of the latent heat of fusion before transfer to an evacuated freezing Dewar flask (3) containing a calibrated volume of 95% ethanol. Samples were controlled cooled at 0.25°C/min from -10°C to -40°C and then transferred to liquid nitrogen for storage. Temperatures were recorded on a Speedomax chart recorder (Leeds and Northrup, North Wales, PA.).

THAWING AND REMOVAL OF THE PROTECTIVE ADDITIVE (FIGURE D-2).

The samples were rapidly thawed in a 37°C water bath at 200°C/min and immediately spun at 450g. After carefully aspirating the supernate, the samples were placed on ice and 1.0 mL of 0.75 M sucrose in supplemented Medium 199 was added. The islets were gently

resuspended in this solution at regular intervals over a 30 min period. One mL of supplemented Medium 199 was then added and the samples were taken from the ice bath and placed at room temperature (25°C). After 5 minutes, an additional 1.0 mL of the isotonic medium was added, followed by 2.0 mL and then 4.0 mL at 5 minute intervals. Islets were resuspended after each addition and half way through the incubation. The samples were then spun at 450g, the supernate was removed and the islets resuspended in supplemented Medium 199 for transplantation (see Plate D-2).

TRANSPLANTATION.

ISO- AND ALLOGRAFTS.

Three thousand WF rat islets were implanted beneath the kidney capsule of diabetic WF or Lewis rats as follows: Islets (1000 to 2000) were pelleted in a conical 250 μL micro-centrifuge tube and the medium was carefully withdrawn using a siliconized glass micropipette and a dissecting microscope. Recipients were anesthetized with 40 mg/kg sodium pentobarbital and a small drop of blood (15-20 μL) was obtained from the recipient's tail with the aid of a 25 μL Accupette pipet (Dade, Miami, FL.) attached to a microcap bulb. The blood was placed on the islets, rapidly mixed with a small glass rod and left at room temperature for 10-15 min to clot. Meanwhile, the left kidney was exposed through a small flank incision. A fine glass rod, inserted through a small nick in the capsule, was used to gently separate the capsule from the kidney surface. The blood clot with embedded islets was picked up with fine forceps and carefully inserted beneath the

capsule with the aid of a dissecting microscope (6X). This procedure is shown in Plate D-3.

Some Lewis rats received a short course of cyclosporin-A. Cyclosporin powder (Sandoz, Basle, Switzerland) was dissolved in Medium Chain Triglyceride oil and absolute ethanol at 50°C to prepare a 20mg/mL solution. It was administered subcutaneously at a dose of 30mg/kg one day prior to transplant, at the time of transplantation and the first two days post-transplant.

XENOGRAFTS.

Mice were anesthetized with Avertin (0.0125 mL/g) and the left kidney was exposed. Five hundred fresh or cryopreserved clean islets, handpicked free of contaminating exocrine tissue, were embedded in a clot prepared from 5-10 μL of recipient blood with the aid of a 10 μL glass capillary tube (Microcaps, Drummond Scientific, Broomall, PA.) attached to a microcap bulb. The clot was picked up with fine forceps and implanted beneath the kidney capsule through a small incision, with the aid of a dissecting microscope. Dextrose, 5% in water, (0.5mL) was given subcutaneously to the mice immediately before transplanting to avoid hypoglycemia induced by insulin release from damaged islets. Animals that died within the first post-operative week or those that did not become normoglycemic were excluded from the study.

At the time of transplantation, some mice received an injection of anti-lymphocyte serum. Rabbit anti-mouse lymphocyte serum (0.2 mL) and rabbit anti-rat lymphocyte serum (0.1 mL) (M.A. Bioproducts, Walkersville, MD.) were mixed and injected via the penile vein five minutes prior to islet implantation. This protocol was modelled after that of Lacy et al. (4).

CONTROLS.

Normal control rats and mice received an injection of 0.1 M acetate buffer without SZ. Both normal and diabetic controls had sham transplants (inserting a blood clot beneath the kidney capsule) two weeks following the induction of diabetes.

METABOLIC PARAMETERS

All islet recipients and controls were housed individually in metabolic cages and were monitored weekly for weight changes, plasma glucose, urine volume and urine glucose. Blood was obtained from the tail in rats and the retroorbital sinus in mice. Two baseline samples of all indices were obtained prior to the administration of SZ. Post-SZ, three samples were obtained within a two week period prior to transplantation. Islet isograft recipients were monitored daily for the first week post-transplant and then weekly for 16 weeks. Nonimmunosuppressed islet allograft recipients were monitored daily post-transplant for two weeks and allograft recipients on cyclosporin were monitored daily for the first three weeks and then biweekly until six weeks post-transplant. Xenograft recipients were monitored daily post-transplant until rejection was evident. Xenograft rejection, following post-transplant normoglycemia, was defined as the first of at least 3 consecutive days of hyperglycemia (plasma glucose exceeding 200 mg/dL).

NEPHRECTOMY AND HISTOLOGY

Graft-bearing kidneys were removed two weeks, six weeks, or four months after transplantation in allograft, immunosuppressed allograft,

and isograft recipients. The kidney was fixed in aqueous Bouins and sectioned at the graft site. Paraffin-embedded tissue sections were stained with insulin-specific aldehyde fuchsin or immunoperoxidase.

STATISTICAL ANALYSIS.

All results are expressed as mean ± standard error of the mean (SEM). Unpaired Student's t-tests were used to compare clinical indices between groups at each time point as well as the ivGTT K values. The criterion for statistical significance was the probability value $P \leq 0.05$.

In the xenograft study, the null hypothesis (H_o) was set as the effect of Group 1 (Fresh) is the same as Group 2 (Cryo) which is the same as Group 3 (Fresh + ALS) and Group 4 (Cryo + ALS).

$$H_o: \gamma_1 = \gamma_2 = \gamma_3 = \gamma_4$$

The Jonckheere-Terpstra test for ordered alternatives (5) is a modification of the Mann-Whitney U test in which the alternative hypothesis (H_a) is that the effect of treatment Group 1 is less than the effect of Group 2 which is less than Group 3. The effect of Group 4 would then be greater than treatment groups 1-3.

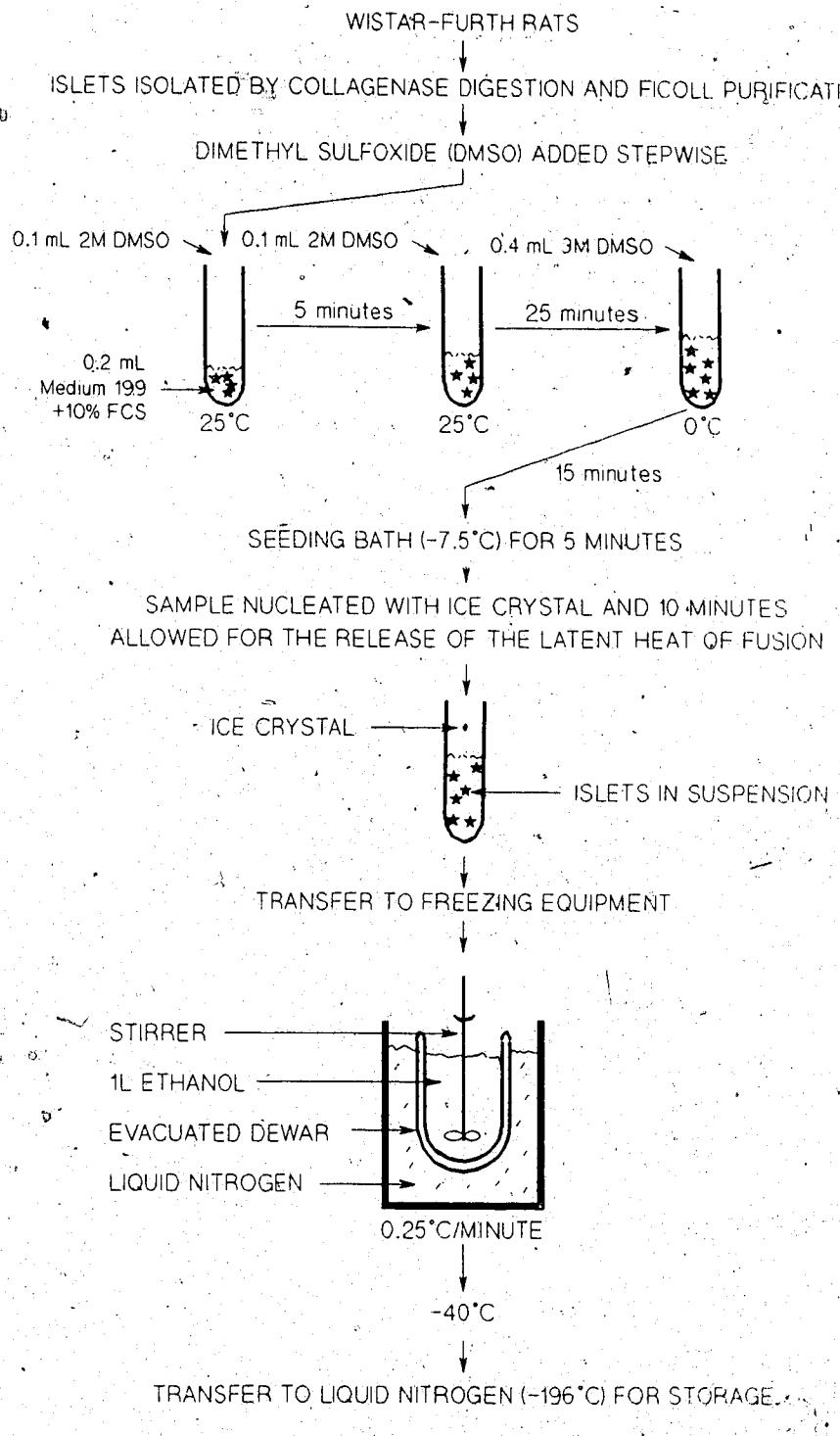


FIGURE D-1. Freezing protocol for rat islets of Langerhans.

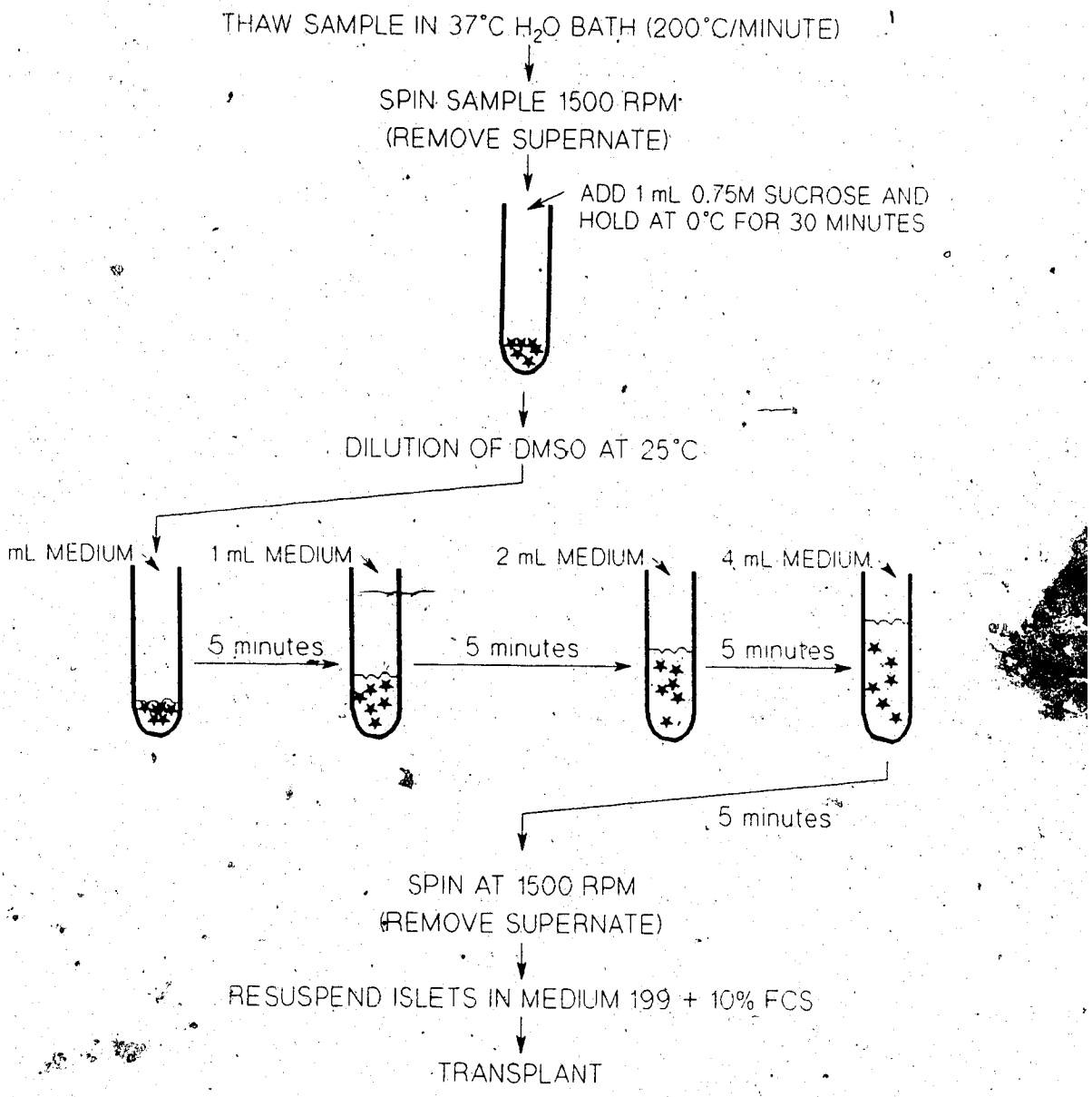
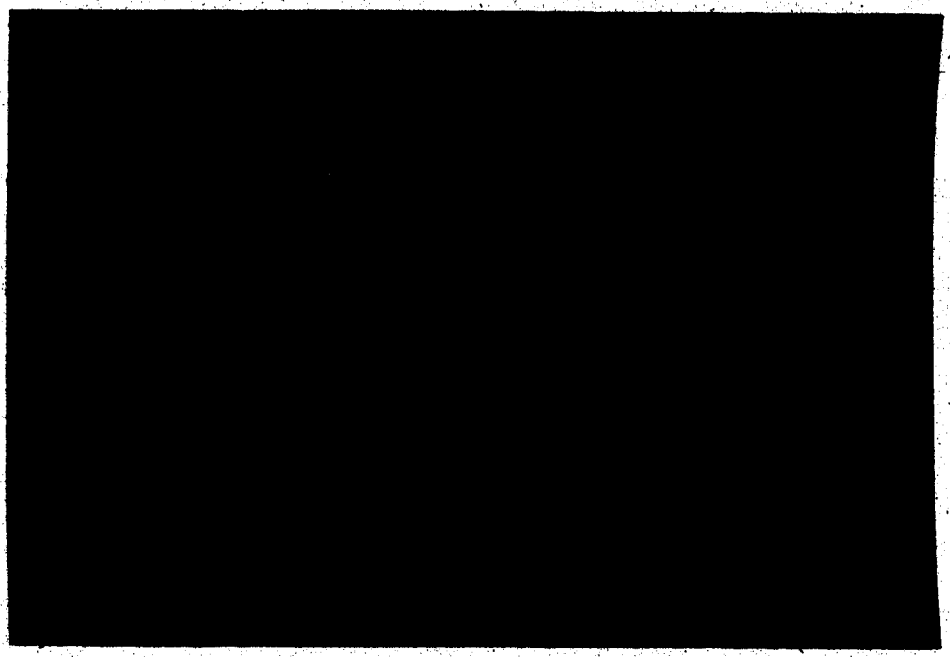


FIGURE D-2. Thawing protocol for rat islets of Langerhans.

(A)



(B)

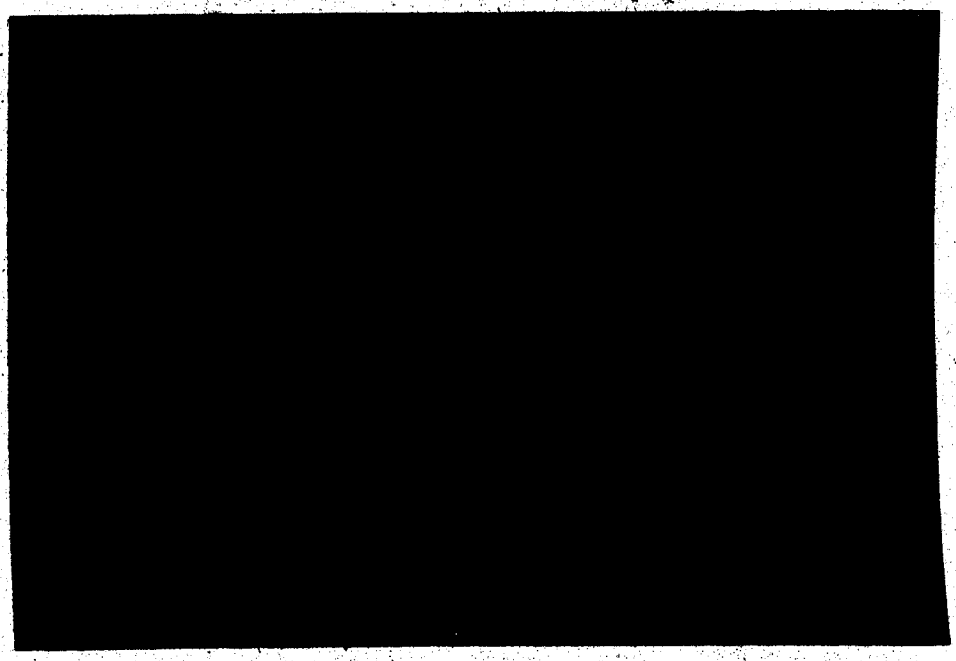


PLATE D-1. Freshly isolated rat islets of Langerhans.
(A) Ficoll preparation with contaminating exocrine tissue.
(B) Preparation handpicked and sized for transplantation.

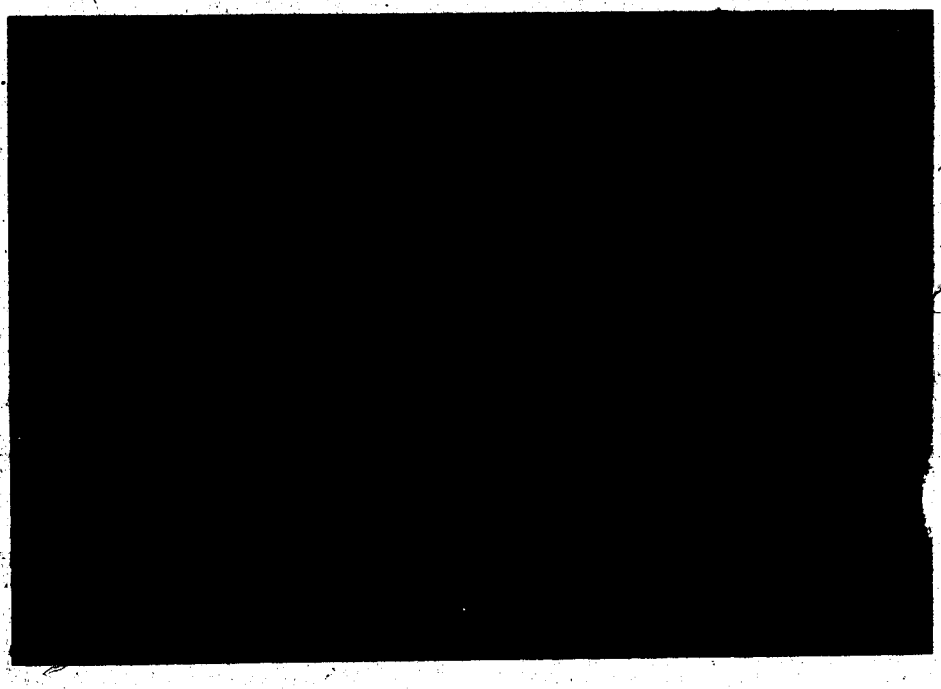


PLATE D-2. Cryopreserved rat islets of Langerhans immediately post-thaw.

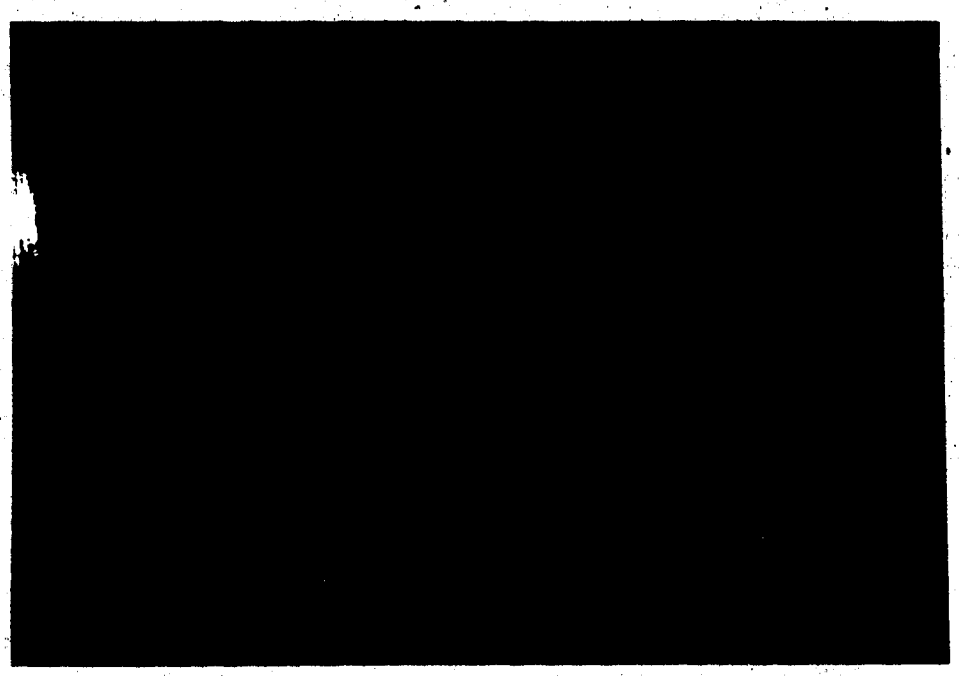


PLATE D-3. Rat islet tranplantation to the renal subcapsular site. Islets are embedded in a blood clot and inserted beneath the kidney capsule with fine forceps.

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