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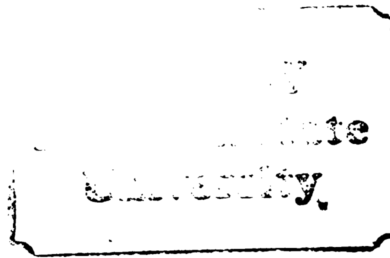

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THE EFFECT OF CYCLOSPORIN A
ON ALLOTRANSPLANTED PANCREATIC ISLETS
IN DLA-MLC COMPATIBLE DOGS

presented by

Malcolm Dexter Williams

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Pathology

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Major professor

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THE EFFECT OF CYCLOSPORIN A ON ALLOTRANSPLANTED PANCREATIC ISLETS IN DLA-MLC COMPATIBLE DOGS

By

Malcolm Dexter Williams

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
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DOCTOR OF PHILOSOPHY

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ABSTRACT

THE EFFECT OF CYCLOSPORIN A
ON ALLOTRANSPLANTED
PANCREATIC ISLETS IN
DLA-MLC COMPATIBLE DOGS

By

Malcolm Dexter Williams

The effectiveness of Cyclosporin A (CyA) in prolonging survival of allogeneic pancreatic islet tissue transplanted to the spleen of totally pancreatectomized dogs was evaluated. Allograft recipients received transplants from DLA-MLC compatible related or unrelated donors. Nineteen dogs were made diabetic by total pancreatectomy. Dispersed pancreatic islet tissue prepared by collagenase digestion was directly implanted into the splenic pulp of the pancreatectomized dogs. Four dogs were given autotransplants and all became euglycemic after a mean (±SE) of 4.5±1.5 days. Three of these animals remained euglycemic until splenectomized 60 days after transplantation. Three nonimmunosuppressed dogs receiving allogeneic transplants from DLA-MLC compatible littermates survived for 21.0±6.6 days and had mean terminal plasma glucose of 399±26.6 mg/dl. Four dogs receiving allogeneic transplants from DLA-MLC compatible littermates were administered CyA (25 mg/kg/day) and none became euglycemic. Animals in this group survived for 25.8±2.3 days and had mean terminal plasma glucose of

365 ± 35.6 mg/dl. Four dogs receiving allogeneic transplants from DLA-MLC compatible littermates were administered CyA (40 mg/kg/day) and 3 of these animals became euglycemic after 8.0 ± 2.0 days. Two of the 3 dogs that became euglycemic remained so until splenectomized at 60 days posttransplant and the third animal was euglycemic until 31 days after transplantation. Each of the 4 dogs in this group survived the length of the study.

Four dogs receiving allogeneic islet tissue from histocompatible unrelated donors were administered CyA (40 mg/kg/day) and none became euglycemic. Animals in this group survived for 26.8 ± 5.4 days and mean terminal plasma glucose was 336 ± 31.1 mg/dl. CyA administered at a dose of 40 mg/kg/day was effective when administered to histocompatible related dogs, but not when administered to histocompatible unrelated dogs. This finding emphasizes the limitations of DLA and MLC as a means of determining histocompatibility and underscores the importance of minor histoincompatibility.

TO
NEIL AND FLORENCE WILLIAMS
(My Parents)

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LIST OF ABBREVIATIONS

CyA	Cyclosporin A
DLA	Dog Lymphocyte Antigen
MLC	Mixed Lymphocyte Culture
HBSS	Hanks' Balanced Salt Solution
IVGTT	Intravenous Glucose Tolerance Test
MHC	Major Histocompatibility Complex
HLA	Human Lymphocyte Antigen

INTRODUCTION

The feasibility of pancreatic islet cell transplantation has been demonstrated by numerous investigators through successful islet tissue autografts in dogs (1-3). In these studies, dispersed pancreatic islet tissue prepared by collagenase digestion without separation of exocrine and endocrine components, ameliorated diabetes when autotransplanted into the splenic pulp or portal vein of totally pancreatectomized dogs. This technique has been successful also in human beings who underwent near total pancreatectomy to relieve the pain of chronic pancreatitis (4, 5). In these cases, however, islet cell mass and function were often compromised prior to transplantation and pancreatic fibrosis hampered islet tissue preparation (6). Isogenic transplantation of isolated islets has been successful in ameliorating experimental diabetes in highly inbred strains of rodents (7-9).

In man and large animals, islet cell allotransplantation has been less successful than iso- and autotransplantation (10-13). Rejection has likely played a major role in this lack of success. Pancreatic islet preparations are highly immunogenic and are extremely susceptible to allograft rejection (14-17), especially across strong histocompatibility barriers. Islets appear to be rejected more rapidly than kidney, heart, and skin following allograft transplantation (14, 18).

Cyclosporin A (CyA) is an immunosuppressive agent which prolongs survival of various organ allografts in a number of species (19-28). Some evidence suggests that CyA reduces the incidence of graft rejection compared with standard methods of immunosuppression (23, 29).

In this study, the efficacy of CyA was evaluated in totally pancreatectomized dogs receiving intrasplenic transplants of dispersed allogeneic pancreatic islet tissue from histocompatible donors. Related and unrelated donor-recipient pairs were used.

LITERATURE REVIEW

The life of the human diabetic patient can be prolonged by exogenous insulin and oral hypoglycemic agents; however, these agents fail to provide the physiologic control of plasma glucose levels that is achieved by a normal functioning pancreas. Glucose sensoring devices implanted into diabetic patients for 24-hour monitoring of plasma glucose levels have demonstrated many periods of hyperglycemia throughout the day; even in patients considered well controlled with insulin (30,31). Even a strict routine of frequent inoculations of short-acting insulin, unlikely to be acceptable or feasible for most patients, does not produce a physiologic response to a glucose load.

Since the discovery of insulin in 1921, the life expectancy of the diabetic patient has been greatly prolonged. However, despite insulin therapy, the morbidity and mortality associated with this disorder continues to be significant. Exogenous insulin can largely prevent death from diabetic ketoacidosis and control the overt symptoms of the disease, but it does not prevent the renal, ocular, and cardiovascular lesions associated with long-term diabetes (30, 32, 33). Although the pathogenesis of these lesions is unclear, considerable evidence supports the hypothesis that they result from disordered carbohydrate metabolism secondary to insufficiency of the pancreatic islets. If this concept is correct, it may be possible to deter or prevent the systemic compli-

cations of diabetes by normalizing carbohydrate metabolism (34, 35, 36). This goal might be achieved (in those diabetic patients with an absolute deficiency of insulin) by providing a physiologically appropriate source of insulin release; such as could be accomplished by successful transplantation of the normal endocrine pancreas.

In 1972, Ballinger and Lacy were the first to demonstrate that isolated adult islets could ameliorate experimental diabetes (7). Using isogeneic strains of rats (no immunologic barrier existed between donor and recipient), they transplanted 400 to 600 islets obtained from multiple donors intraperitoneally to recipients with streptozotocin-induced diabetes. Isolated islets were obtained by digesting minced donor pancreata with collagenase, and then separating the islets from the exocrine tissue by centrifugation in a discontinuous gradient of Ficoll.

Many different sites of islet transplantation in the rodent have been successful (7,37,38,39,40,41), including placement intraperitoneally, subcutaneously, and subcapsularly in the kidney, as well as placement in the thoracic cavity, the testis, the spleen, and, most effective of all, the portal venous system.

Islet transplantation techniques that were effective in the rodent model were less successful when adapted to higher animals such as pigs, dogs, monkeys, and man (42,43,44,45,46). In these cases, the yield of isolated islets was low due to the relative inefficiency of the collagenase digestion-Ficoll gradient separation technique when applied to the pancreata of higher animals; the tough, fibrous, compact structure of the adult pancreas in these instances made collagenase digestion and

separation of islets difficult and this was responsible for the low yield of islets (in humans the average yield was only 5 percent). Other factors that contributed to islet transplantation being less successful in higher animals than in rodents were allograft rejection, and because the lack of large animal inbred strains made it difficult to use multiple donors to compensate for the low yield of islets from one pancreas.

In the initial stages of islet transplantation study, the separation of islet tissue from exocrine components (islet purification) was thought to be necessary for successful transplantation. As discussed above, specific islet purification techniques were successful in rodents, but relatively inefficient in higher animals, including man.

In 1976, Mirkovitch and Campiche (1) made a major contribution to the field of islet transplantation when they demonstrated in the dog that multiple donors and islet cell purification were not necessary for successful transplantation. In this study, dispersed collagenase-digested islet tissue that had not undergone separation of endocrine and exocrine components, ameliorated diabetes when autotransplanted into the spleen of totally pancreatectomized dogs. By eliminating the steps necessary for islet purification, a larger quantity of islet tissue was available for transplantation.

The following is a description of the islet tissue preparation and implantation techniques of Mirkovitch and Campiche. After laparotomy on the experimental subject, a ligature was tied at the junction of the horizontal and vertical part of the pancreas. The horizontal part of the gland was infiltrated in situ through a cannula in the excretory duct or by direct puncture of the tissue with a fine needle. The infiltration fluid was modified Ringer's lactate solution containing

1 mg/ml of collagenase; 20-30 ml were injected depending on the quantities of tissue. The distended portion of the pancreas was removed, cut into 2-4 mm pieces, and rinsed in 300 ml of modified Ringer's solution without collagenase. After sedimentation for about 45 seconds, the supernatant was removed by aspiration, the volume of the sediment was measured and 5 mg of collagenase were added for each ml of tissue. The tissue was then placed in a gently-shaking water bath for 15 minutes at 37° C. After this period of incubation, modified Ringer's solution was added up to a total volume of 100 ml, and the suspension was centrifuged for 5 minutes at 2,100 g. Following removal of the supernatant, the sediment was resuspended and recentrifuged under the same conditions. The supernatant was discarded and the sediment (5.5-14.0 ml), was placed in a syringe and diluted to a final volume of 20 ml. To implant the dispersed islet tissue, a branch of the splenic vein draining the upper half of the organ, was dissected, tied, and cannulated with a 2 mm cannula. This was carefully advanced into the middle of the splenic tissue, and passed into the lower half of the organ. The syringe containing the prepared islet tissue was connected to the cannula and emptied slowly to ensure adequate dispersion. After the injection, the cannula was removed, splenic circulation was reestablished and the operation was terminated with complete removal of the rest of the pancreas. Postoperatively, the animals received no anti-diabetic treatment.

In the monumental study by Mirkovitch and Campiche, 7 dogs were given intrasplenic autografts of dispersed, unpurified islet tissue. Rejection obviously was not a factor since these were autotransplant situations. Mongrel animals of both sexes, weighing 15-21 kg, were used.

Diabetes was ameliorated in all of the transplanted dogs. Plasma glucose concentration increased in all 7 animals during the first 3 postoperative days, with the highest value of 286 mg/dl being recorded in 1 dog on the third day. After the third day, hyperglycemia progressively decreased, and from the tenth postoperative day onwards, all 7 animals remained euglycemic with plasma glucose levels between 80 and 120 mg/dl. Glucose loading tests were performed between the 29th and 38th postoperative days. The pattern and values of plasma glucose did not differ from those of a control group of nonoperated dogs. Five animals were euglycemic 1 hour after glucose injection, while the other 2 had plasma glucose values of 135 and 137 mg/dl. Plasma insulin concentrations promptly increased following administration of a glucose load in all 7 dogs. On the 66th postoperative day, 1 euglycemic animal died of undetermined causes. Splenectomies were performed on the 6 surviving euglycemic dogs (plasma glucose values between 91 and 117 mg/dl), 72 to 78 days after the islet autotransplantation; the spleen was the site of islet tissue implantation. In all 6 animals, splenectomy was followed by the prompt appearance of hyperglycemia of over 300 mg/dl, which lasted until their deaths 4-13 days later. Histologically, numerous fragments of pancreatic tissue were observed to be dispersed throughout the splenic pulp. Exocrine tissue varied in appearance from normal looking acini with zymogen granules to vesicles lined by flattened cuboidal epithelium. Small groups of pale epithelial cells were observed which were highly suggestive of islets. Electron microscopy confirmed the presence of both exocrine and endocrine pancreatic tissue.

Another major contribution to the field of islet transplantation was made by Kolb, Ruckert and Largiader in 1977 (3). Using dogs, they autotransplanted dispersed pancreatic islet tissue that had not undergone purification steps, into the liver (via the portal vein) in some animals and into the spleen in others. The experiments were performed on 10 mongrel dogs of both sexes, weighing 15-25 kg. Five received an intraportal and 5 an intrasplenic autotransplant. Plasma glucose concentrations rose slightly during the first and second postoperative days with both types of transplants but remained below 200 mg/dl and reached preoperative values within the first week. Glucose tolerance tests after 4 weeks showed slightly higher peak values and a slower decline of glucose levels as compared to normal controls. No significant functional difference was observed between the 2 transplant groups (intraportal and intrasplenic). All animals were sacrificed in good condition 4 to 6 weeks after transplantation. Pancreatic exocrine and endocrine fragments were observed in serial sections of the liver within minor portal venous branches or capillaries in the intraportally transplanted animals. In the dogs with intrasplenic transplants, macroscopically the spleen sometimes showed a patchy thickening of the capsule. Histologically, endocrine as well as exocrine fragments were observed intrasplenically.

Further islet tissue autotransplantation experiments in dogs were performed by Kretschmer et al. (2) as well as by Schulak et al. (47). The results of these experiments were similar to those of the experiments in dogs already discussed.

In 1977, Najarian et al., in their initial experience with allotransplantation of islet tissue in humans with diabetes mellitus (45),

performed 10 islet transplant procedures in 7 insulin dependent diabetic patients. Each patient had received a renal allograft 10-36 months previously for treatment of end stage diabetic nephropathy. Standard immunosuppressive therapy was administered to all 7 patients. Because a reliable technique of islet preservation was not available, the islets were transplanted immediately after processing of the pancreas. Therefore, histocompatibility testing was not used as a basis for recipient selection, and all donor-recipient pairs were poorly matched. The pancreas of a brain-dead cadaver was the source of tissue for each transplant. Six donors were infants, 2 days to 1 year old, and 4 donors were adults. At the time of this study, the authors believed that a purified islet preparation was required or that exocrine enzymes must be completely eliminated prior to transplantation. Highly purified islet tissue was obtained from the 4 adult pancreata by the collagenase digestion-Ficoll gradient islet isolation technique (41,42,45). The 6 infant pancreata were simply dispersed by mincing and collagenase digestion; as the neonatal pancreas has a low exocrine enzyme content, no attempts were made to separate endocrine and exocrine components. After preparation of the donor pancreata, the islet tissue was implanted intraperitoneally in 5 instances, into a muscle pocket in 1 instance, and infused into the portal vein in 4 instances. The initial human islet transplants by Najarian et al., though not ideal, were successful enough to suggest that the concept merited further pursuit. Intervals of reduced insulin administration occurred in the 4 transplants via the portal vein, and in 3 of the 5 intraperitoneal transplants. The duration of decreased insulin administration varied between 2 and 9 weeks in 6 of the transplant patients. The reduction in insulin dose

was sustained in excess of 18 months in 1 patient. The reductions in insulin dosage were not associated with decreased diabetic control, as the mean plasma glucose values and the mean 24 hour urinary glucose excretion values were improved in reference to pretransplant levels. A decrease in insulin dose suggests islet function if the control of diabetes is as good or better than the control before transplantation.

In these preliminary trials by Najarian, et al., low islet yields were obtained from the adult pancreata, and the neonatal pancreata were subjected to prolonged collagenase digestion; a technique that destroyed more than 70 percent of the islet tissue. The minimal collagenase digestion technique for dispersal of the human neonatal pancreas had not yet been developed at this time. Because of the small quantities of islet tissue transplanted, it was difficult to determine if graft failure was caused by rejection, inadequate islet mass, or technical problems.

In 1978, the team of Sutherland, Matas, and Najarian performed the first successful islet transplantation procedure in humans (46). They used an adaptation of the unpurified islet tissue preparation technique developed by Mirkovitch and Campiche. By eliminating the steps necessary for islet purification, a larger quantity of islet tissue was available for transplantation. In this study, Sutherland et al. autotransplanted dispersed islet tissue into the liver of a woman with chronic pancreatitis who underwent near total pancreatectomy for incapacitating, intractable pain. Total or near total pancreatectomy can relieve the pain of pancreatitis, but is rarely applied because the metabolic consequences are so severe.

The prepared tissue containing the patients' own islets was returned to the operating room 1 1/2 hours after pancreatectomy. The

tissue was slowly embolized to the liver by infusion into the mesenteric vein. During the period between pancreatectomy and autotransplantation, and for the first 24 hours after surgery, plasma glucose levels ranged from 229 to 352 mg/dl. Plasma glucose levels ranged between 144 to 284 mg/dl for the next 4 days and then declined to between 100 and 195 mg/dl for the next 2 weeks. Beginning 3 weeks after transplantation, fasting plasma glucose levels ranged from 77 to 104 mg/dl, and plasma glucose levels two hours after a meal ranged from 110 to 146 mg/dl. The patient did not receive insulin at any time after transplantation.

In 1979, the observations made in the above report were confirmed by further experimental trials by Najarian, Sutherland, et al. (48). In this study, dispersed islet tissue was autotransplanted into the liver of 3 patients being treated for chronic pancreatitis. Once again, islet tissue was prepared using an adaptation of the technique of Mirkovitch and Campiche.

The course of the first patient was initially reported in 1978 and is described in detail in the above study (46). Briefly, the plasma glucose levels were evaluated for the first few weeks after transplantation (maximum level 352 mg/dl), but the patient never required insulin. By 3 weeks after transplantation, plasma glucose had declined and ranged from 77 to 104 mg/dl in the fasting state and from 110 to 146 mg/dl after eating. The patient remained euglycemic for at least 18 months.

In the second patient, plasma glucose levels were greater than 400 mg/dl immediately posttransplant, requiring 22 U of insulin be administered during the first 24 hours. Plasma glucose then stabilized at less than 200 mg/dl, and the patient received no insulin for the next 24 hours, even though 10 percent dextrose was being administered by

continuous infusion. For the next 7 days, continuous 10 percent dextrose infusion was administered and 1-2 U of insulin was given every 1-4 hours to maintain plasma glucose levels between 100 and 200 mg/dl. Unfortunately, on the seventh postoperative day, the patient suddenly developed abdominal pain, and an exploratory laparotomy revealed an acute perforation of the transverse colon. The patient developed severe peritonitis and died 3 days later from sepsis. An autopsy was performed and histologic examination of the liver showed numerous islets located in the portal venule vascular spaces. A Masson-trichrome stain showed granulated viable endocrine cells with red blood cells and capillaries within the islets, indicating that neovascularization had occurred at the time of death (10 days after transplantation). Immunoperoxidase staining confirmed the presence of insulin-containing beta cells and glucagon-containing alpha cells within the transplanted islets. Due to the untimely death of the patient, metabolic studies and long-term assessment of islet function was not possible. However, the well preserved state of islets in the liver was remarkable and documented the ability of free islet tissue autografts to survive in the human liver.

The third patient was maintained on intravenous dextrose-containing fluids for 25 days following autotransplantation; during this interval, exogenous insulin (mean dose 18$\pm$3 U/day) was administered in an attempt to keep plasma glucose concentration below 200 mg/dl. Insulin administration was completely stopped after termination of intravenous feedings and initiation of oral feedings. The patient received no insulin injections after that time and was euglycemic for at least 3 months posttransplant (up to the time of the report).

The most important aspect of the autotransplant studies reviewed above was the establishment of the technical feasibility of islet transplantation in humans. A large quantity of unpurified islet tissue was infused into the portal venous system of patients who had undergone pancreatectomy for treatment of chronic pancreatitis, and diabetes was obviated. Because these were autotransplant situations, the results were not influenced by the possibility of rejection.

In man and higher animals, the results of islet tissue allotransplantation have been disappointing (10,11,12,13). According to the International Transplantation Registery, since the inception of human islet transplantation attempts through the end of 1981, seventy-six allotransplant procedures in 71 diabetic patients were reported; of these, only 4 patients became insulin independent for sustain periods of time. The major factor in the lack of success of islet allotransplantation has most likely been rejection. Pancreatic islets are highly immunogenic and are extremely susceptible to allograft rejection (14,15,17), especially across strong histocompatibility barriers. In untreated hosts, allogeneic islets appeared to be rejected more rapidly than other tissues (14, 16, 18), and immunosuppressive regimens that prolonged the survival of skin, kidney, and heart allografts were often ineffective in prolonging the survival of islet allografts ( ).

The major factor limiting wide application of islet transplantation has been the difficulty in preventing allograft rejection. Improved methods for preventing rejection must be developed before islet allotransplantation realizes its full potential. This problem could be minimized by thorough histocompatibility testing to reduce donor-recipient antigen differences, and by more effective methods of immunosuppression.

That the rejection phenomena is immunologic in nature, implies that the lymphoid system must recognize foreign cell-surface antigens on the donor tissue and mount an immune response against them. These cell-surface antigens are called histocompatibility antigens (transplantation antigens). Although each animal species possesses a wide variety of histocompatibility antigens, they are not all equally immunogenic. Therefore, some are considerably more important than others and grafts differing from the host in these "major" antigens are rejected much more promptly than grafts differing in "minor" antigens. These major histocompatibility systems (49,50-52,59) include the H-2 system in mice, the HLA (human lymphocyte antigen) system in man, and the DLA (dog lymphocyte antigen) system in dogs.

The major histocompatibility antigens are glycoproteins firmly attached to almost all cell surfaces; lymphocytes possess the highest content of these antigens. It has been suggested that each antigen molecule consists of 4 peptide chains, 2 of which are "heavy" and 2 "light". They thus generally resemble immunoglobulin molecules in structure and their characteristic antigenicity is primarily associated with antigenic determinants on their heavy chains.

Histocompatibility antigens are inherited and are coded for by genes of the major histocompatibility complex (MHC). The MHC consists of a number of closely linked loci on a single chromosome, that are polymorphic in expression. This system is found on chromosome 6 in humans, and on chromosome 17 in mice.

In the dog, the MHC is termed the DLA system. Genes of the DLA system control the expression of antigenic determinants on parenchymal cells of the body; this system consists of 4 closely linked loci. Of

these loci, 3, i.e., DLA-A, DLA-B, and DLA-C, code for antigens that are serologically defined (53-56), and 1, i.e., the DLA-D locus codes for antigens that are defined by cell-mediated assay (57,58). The standard serologic assay is a complement-dependent lymphocytotoxicity test which generally uses rabbit serum as the source of complement. In this test, lymphocytes from the animal to be typed are incubated with specific antisera containing antibodies against each individual canine histocompatibility antigen; this is followed by incubation with complement. Antigen-bearing lymphocytes are killed in different antisera on the basis of their specificities. Cell-mediated detection of histocompatibility determinants is directed at the detection of those determinants for which there are no serologic methods or reagents. This form of assay is done by mixed lymphocyte culture (MLC). In the MLC test, lymphocytes from recipient and donor animals are mixed. If incompatible, each cell population will be stimulated to divide by the presence of foreign antigens, and this division is detected by measuring the rate of uptake of tritium (^{3}H) labelled thymidine by the cultured cells.

There is a direct relationship between allograft survival time in the dog and DLA compatibility, and if transplantation is considered in this species, attempts should be made to ensure DLA compatibility between donor and recipient.

However, no matter how histocompatible similar siblings or unrelated individuals are, the MHC is only an extremely small component of those individuals' genetic makeup. There are numerous other genetic differences (minor histoincompatibilities), that in transplantation translate into antigen differences. Ideally, most of these "other" antigenic differences are abrogated by immunosuppression. Unfortunately,

as mentioned earlier, standard immunosuppressive regimens (usually incorporating steroids) that were effective in transplantation of other tissues, were often ineffective in prolonging islet allograft survival. More efficient methods of immunosuppression would significantly facilitate the potential success of islet allotransplantation. A new immunosuppressive compound, Cyclosporin A (CyA), offers a great deal of promise in accomplishing this goal.

CyA, the most promising immunosuppressive agent of recent years, is an extract from the fungi Cylindrocarpon lucidum and Trichoderma polysporum. It was discovered in 1972 in the laboratories of Sandoz in Switzerland during a search for biologically produced antifungal agents. Although CyA proved to have only mild antifungal activity, it was found to have a number of interesting influences on the immune system. The drug has been shown to cause suppression of both humoral and cellular immunity (60-64.).

CyA has been demonstrated to prolong survival of organ allografts in all species tested thus far (19-28). It prolonged heart allografts in rats and pigs (65, 66); and allowed transplantation of allogeneic skin grafts in dogs (20) and in rabbits (67). CyA has also been demonstrated to be a potent suppressor of rejection of kidney allografts in rabbits (24), dogs (68), and rats (27). In addition to prolonging the survival of organ allografts in a number of species, CyA has also been found to inhibit the development of graft-versus-host disease, delayed skin hypersensitivity, and chronic inflammatory reactions (61). Humoral responses to various antigens were also suppressible by CyA (61,62,69). These in vivo effects were paralleled by equivalent effect in vitro, and CyA has been shown to inhibit blast transformation, proliferation, and

antibody formation in response to stimulation by mitogens and major histocompatibility complex alloantigens (69,70).

The specific mechanism and cellular locus for the immunosuppressive action of CyA is unknown. Initial reports suggested that the inhibitory effects of the drug were limited to the T lymphocyte subset; the drug was shown to suppress B lymphocyte proliferation or antibody formation only in the case of T-dependent responses (61, 62, 69, 73). However, more recent reports have suggested that CyA can exert its immunosuppressive effects independently of T lymphocytes (63,71,72). These studies found that CyA was equally suppressive for the blastogenic responses of human B and T lymphocytes, and that the drug inhibited equally well the proliferative responses to both T-dependent and T-independent mitogens. The cellular locus for the immunosuppressive effect of CyA thus remains unclear. Whatever its locus of action, CyA did not seem to exert any permanent effect on its target cells; suppression of humoral or T lymphocyte mediated responses, both in vivo and in vitro, was reversible after withdrawal of the drug (73,74,75).

Results of several clinical trials using CyA suggested that the incidence of graft rejection was reduced as compared with conventional immunosuppressive regimens (azathioprine, corticosteroids, and antilymphocyte immunoglobulins) (23,29,76,77). Other advantages of CyA over conventional methods of immunosuppression is that it is nonmyelosuppressive, relatively nontoxic, and numerous studies have demonstrated that the drug can maintain long-term allograft function when used alone as a single agent. In reference to pancreatic islet allotransplantation, another important advantage of using CyA for immunosuppression, rather than conventional methods which incorporate corticosteroids, is that the

high dose of steroids given to islet recipients may produce a diabetogenic effect sufficient to mask any graft function that may occur before rejection; this potential problem is avoided by using CyA.

Even though CyA is relatively nontoxic in comparison with conventional immunosuppressive regimens, several adverse effects have been associated with this agent; the most important being nephrotoxicity. However, this condition was usually readily reversible when the drug dosage was lowered, and not surprisingly, was more dangerous when CyA was administered in conjunction with aminoglycosides and other nephrotoxic drugs such as the fungistat amphotericin B. Other, less frequent side effects have included hepatotoxicity, anorexia, nausea, depression, tremors, skin thickening, hirsutism, hypertrophy of gums and gingivitis. These conditions were also usually readily reversible by decreasing CyA dosage. Some investigators have reported an increased incidence of lymphomas in both man and nonhuman primates that were treated with CyA.

In summary, transplantation of normal islet cells into an insulin-dependent, ketosis-prone diabetic patient is the most logical and comprehensive approach to treatment of the disease. The technical feasibility of pancreatic islet cell transplantation has been demonstrated through successful autotransplants in man and higher animals, and through isotransplants in rodents. The discouraging experiences in islet tissue allotransplantation are most likely primarily due to rejection. The problem of allograft rejection can be minimized by thorough histocompatibility testing to select optimal donor-recipient pairs, and by improved immunosuppression using Cyclosporin A. If the rejection phenomena is adequately addressed, transplantation of pancreatic islet tissue could be a safe and effective treatment for insulin-dependent diabetes mellitus.

MATERIALS AND METHODS

Animals

Conditioned Labrador Retrievers, Siberian Huskies, Beagles, Golden Retrievers, and a variety of mixed-breed dogs weighing between 10 and 25 kilograms (kg) were used. The dogs were free of intestinal parasites and heartworms and were vaccinated against distemper, *Leptospira,* hepatitis, and parvovirus.

Histocompatibility Testing

Serologically defined dog lymphocyte antigens (DLA)-A, B and C were determined by two-stage lymphocytotoxicity method (53-56). Cell-mediated assays were performed using mixed lymphocyte culture (MLC) technique (58). Donor-recipient pairs were then selected on the basis of DLA identity and nonstimulation of recipient lymphocytes by donor cells in the MLC.

Immunosuppressive Therapy

Cyclosporin A (CyA), dissolved in olive oil, was orally administered at doses of 25 mg/kg/day or 40 mg/kg/day. When the drug was used at the latter rate, it was given in divided doses. To facilitate absorption of the drug, 3 five grain Viokase tablets (A. H. Robbins Co.) were administered immediately before the CyA was given. In those allograft recipients that were immunosuppressed, therapy was initiated 2 days before transplantation.

Pancreatectomy and Preparation of Dispersed Pancreatic Fragments

General anesthesia was induced with thiamylal sodium and maintained with halothane, oxygen, and nitrous oxide. The pancreas was exposed through a ventral midline incision extending from the xiphoid process to the caudal umbilicus. The left limb and body of the pancreas were quickly removed and immediately immersed in iced Hanks' balanced salt solution (HBSS). This segment was taken directly to the laboratory for preparation. The right limb of the pancreas was meticulously dissected in order to preserve the pancreaticoduodenal vessels and bile duct. Resection of this segment was usually completed within 30 minutes after removal of the initial segment. The tissue was placed in iced HBSS and promptly transported to the laboratory for preparation.

The pancreas was weighed and then distended by direct infiltration with cold (4 $^{\circ}$C) HBSS. The edematous organ was freed of major blood vessels and connective tissue and then manually minced into fragments with a maximal diameter of 2 mm. The diced tissue was washed 3 times by sedimentation in cold HBSS. The tissue was then suspended in 37 $^{\circ}$C HBSS to a total volume of 100-125 ml and digested for 20 minutes in a shaking water bath (37 $^{\circ}$C) with 1,350 units of collagenase (Worthington, Type IV) per gram of tissue. After digestion, the tissue was washed 4 times by sedimentation in cold HBSS. The tissue was then placed into 50 ml centrifuge tubes and pelleted by centrifugation at 1,000 rpm for 1 minute. The digested tissue was adjusted in cold HBSS to a total volume of 35-50 ml and placed in a 50 cc syringe for implantation into the spleen of recipient animals.

Islet Tissue Implantation

In each recipient, the splenic capsule was directly punctured with a 14 gauge 5.5 inch needle catheter which was inserted parallel to the longitudinal axis of the spleen. The syringe was attached, and the tissue was injected as the catheter was slowly withdrawn. The catheter was passed at least twice in order to achieve wide dispersal of pancreatic fragments throughout the splenic pulp. Hemostasis was achieved at each of the puncture sites by placing a 5-0 chromic gut mattress suture over an absorbable sponge.

Experimental Groups

Each dog in Groups 2, 3, 4, 5 and 6 was made diabetic by total pancreatectomy and received intrasplenic transplants of autologous or allogeneic dispersed pancreatic islet tissue.

Group 1 (n=16). Normal dogs were used to establish the ranges of normal fasting plasma glucose values and normal intravenous glucose tolerance test (IVGTT) curves.

Group 2 (n=4). Received autologous dispersed pancreatic islet tissue.

Group 3 (n=3). Received allogeneic islet tissue from DLA-MLC compatible littermates. Immunosuppressive treatment was not administered.

Group 4 (n=4). Received allogeneic islets from DLA-MLC compatible littermates. CyA was administered at a dose of 25 mg/kg/day.

Group 5 (n=4). Received allogeneic islet tissue from DLA-MLC compatible littermates. CyA was administered at a dose of 40 mg/kg/day.

Group 6 (n=4). Received allogeneic islet tissue from unrelated DLA-MLC compatible donors. CyA was administered at a dose of 40 mg/kg/day.

Postoperative Care

Dogs received Ringer's lactate solution intravenously until they had returned to normal oral alimentation. All animals were treated with ampicillin (10 mg/lb, TID) subcutaneously for the first 5 postoperative days. No oral intake was allowed for the first 1 to 2 days following surgery. After this time, water was given in small amounts, and if no vomiting occurred, small quantities of semisolid food were offered every 4 hours. For the first 24 to 48 postoperative hours, plasma glucose concentrations were determined every 4 hours, and if values fell below 60 mg/dl, intravenous boluses of 50% dextrose were administered in amounts adequate to establish euglycemia. Normal oral alimentation had usually returned by 3 to 4 days following surgery and dogs were then fed a standard kennel diet with pancreatic extract (Viokase, A. H. Robbins Co.) added to compensate for the exocrine deficiency produced by pancreatectomy.

Metabolic Studies

Normal carbohydrate metabolism was verified in all donors and recipients prior to surgery by multiple fasting plasma glucose determinations and 1 intravenous glucose tolerance test (IVGTT). Intravenous glucose tolerance tests were performed using 0.5 g of 50% gluocose per kg of body weight. The glucose bolus was administered as a rapid injection into the cephalic vein. Peripheral blood samples were collected before injection (0 minutes) and at 5,]0, 15, 30, 45, 60, 90 and 120 minutes thereafter. Samples were assayed for glucose and insulin concentrations. K values (percentage of decline of plasma glucose concentration per minute) were calculated from the 5, 10, 15, 30 and 45 minute

postinjection plasma glucose values using the method of least squares. Plasma glucose concentrations were measured by the glucose oxidase method using a Beckman Glucose Analyzer 2. Euglycemia was defined as a plasma glucose level of less than 150 mg/dl after a 12 hour fast. Plasma insulin was measured by radioimmunoassay using Corning's IMMOPHASE™ insulin radioimmunoassay test system.

Fasting plasma glucose concentrations were determined in recipient animals daily for 2 weeks following transplantation and a minimum of 3 times weekly thereafter. In surviving animals, IVGTTs were performed at 3 and 8 weeks after transplantation.

Termination of Experimental Animals

Surviving dogs were studied for a maximum of 60 days, after which time splenectomies were performed. Multiple sections of spleen were fixed in 10% buffered formalin and Bouin's solution for histologic examination. Daily fasting plasma glucose determinations and 1 IVGTT were done on each splenectomized subject. Animals were sacrified 3 to 5 days after splenectomy.

Other criteria for terminating study on individual animals were death and sacrifice because of debilitation with severe hyperglycemia, ketoacidosis, weight loss and inappetence.

Histologic Studies

Necropsy examinations were performed and multiple sections of tissue from lungs, heart, kidneys, liver, lymph nodes, and spleen were fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Sections of spleen were also

fixed in Bouin's solution and stained by immunoperoxidase technique (79, 80) for identification of insulin-containing beta cells.

Data Analysis

One-way analysis of variance (ANOVA) and the Walker-Duncan Adaptive Procedure were used to analyze the data. All evaluations were conducted at a probability level of 0.05.

RESULTS

Survival and Metabolic Data

Group 1. Normal dogs had mean (±SE) fasting plasma glucose values of 82±2.42 mg/dl and mean K values of 2.9±0.2%. The mean of fasting basal plasma insulin values prior to IVGTTs was 19±1.6 μU/ml and following administration of a glucose load mean plasma insulin values, peaked at 68±9.0 μU/ml.

Group 2. The 4 dogs receiving autologous islet tissue became euglycemic 4.5±1.5 days after transplantation. Three animals remained euglycemic until splenectomized 60 days following surgery. The dog that did not maintain normal fasting plasma glucose for 60 days developed hyperglycemia from day 35 to day 49. Daily fasting plasma glucose during this period was 202±16.1 mg/dl (additional information given in Table 1). The mean K value of the 4 animals 3 weeks posttransplant was 1.5±0.2%. At 8 weeks, the mean K value for the 3 dogs that were euglycemic for 60 days was 1.8±0.3%, indicating a significant impairment of glucose degradation when compared to the normal dogs ($P<0.05$), although the animals maintained euglycemia.

During the 8 week IVGTT, plasma insulin increased from a basal concentration of 26±2.2 μU/ml to a peak of 29±3.2 μU/ml.

Splenectomies were performed at 60 days on the 3 dogs that maintained euglycemia for the entire posttransplant period; mean plasma

glucose was 97±6.5 mg/dl before splenectomy. Hyperglycemia developed in all of these animals and the mean K value of IVGTTs performed 2 days later was 0.4±0.03%. The dogs were sacrified 3 to 5 days after splenectomy. Mean plasma glucose values prior to sacrifice were 395±5.6 mg/dl.

Group 3. Three nonimmunosuppressed dogs received allogeneic islet tissue from histocompatible littermates and 2 of them never became euglycemic; the other animal had a short duration of euglycemia from day 6 to day 10. The mean survival time was 21±6.6 days. The mean K value of 2 surviving dogs 3 weeks posttransplant was 0.3±0.0%. During IVGTT plasma insulin increased from a basal concentration of 11±1.0 μU/ml to a peak of 12±0.0 μU/ml. The mean plasma glucose of this group at the time of the death was 399±26.6 mg/dl.

Group 4. Four dogs receiving allogeneic islet tissue from histocompatible littermates were administered CyA (25 mg/kg/day) and none became euglycemic. The mean survival time was 25.8±2.3 days. The mean K value of 3 surviving dogs 3 weeks posttransplant was 0.4±0.1%. During IVGTT, plasma insulin increased from a basal concentration of 8±0.5 μU/ml to a peak of 16±8.5 μU/ml. The mean plasma glucose of this group at the time of death was 365±35.6 mg/dl.

Group 5. Four dogs receiving allogeneic islet tissue from histocompatible littermates were given CyA (40 mg/kg/day) and 3 animals became euglycemic 8.0±2.0 days after transplantation. Two animals remained euglycemic until splenectomized 60 days following surgery. The dog that did not maintain normal fasting plasma glucose for 60 days became hyperglycemic on day 31 and remained so for the duration of the study. Daily fasting plasma glucose from day 31 to day 60 was

218±5.1 mg/dl (additional information given in Table 1). At 3 weeks posttransplant, the mean K value of the 3 animals that were euglycemic was 0.9±0.2%. At 8 weeks the mean K value for 2 dogs that were euglycemic was 1.7±0.2%; this value was significantly lower than that of the normal dogs ($P<0.05$), but not significantly different from Group 2 at 8 weeks.

During the 8 week IVGTT, plasma insulin increased from a basal concentration of 18±2.5 μU/ml to a peak of 24±0.5 μU/ml.

Splenectomies were performed at 60 days on the 2 dogs that maintained euglycemia for the entire posttransplant period; mean plasma glucose was 97±8.0 mg/dl before surgery. Hyperglycemia developed in both animals and the mean K value of IVGTTs performed 2 days later was 0.4±0.0%. The dogs were sacrificed 4 days after splenectomy with final mean plasma glucose values of 356±7.0 mg/dl.

Group 6. Four dogs receiving allogeneic islet tissue from histocompatible unrelated donors were given CyA (40 mg/kg/day) and none became euglycemic. The mean survival time was 26.8±5.4 days. There was no significant difference between the survival times of Groups 3, 4 and 6 ($P<0.05$). The mean K value of 3 surviving dogs 3 weeks posttransplant was 0.5±0.1%. During IVGTTs plasma insulin increased from a basal concentration of 7±1.3 μU/ml to a peak of 9±3.2 μU/ml. The mean plasma glucose of this group at the time of death was 336±31.1 mg/dl.

Histologic Examination

Moderate to severe fatty infiltration was observed in sections of liver from all transplanted dogs; including those animals that became diabetic following splenectomy. There was no consistent evidence of toxicity in any of the tissues examined. In group 2 (autografts), numerous well preserved pancreatic fragments were dispersed throughout all the spleens; in some areas associated with moderate fibrosis. Immuno-operoxidase staining readily identified beta cells within the transplanted tissue. In group 5 (related allografts, CyA at 40 mg/kg/day), the appearance of the spleens was similar to that described in group 2 in the 3 animals that achieved euglycemia. In the 1 animal that never became euglycemic, pancreatic fragments were seen infrequently; beta cells were not identified. In group 3 (related allografts, no CyA), all the spleens had extensive areas of necrosis, and severe, widespread fibrosis. Pancreatic fragments were not identifiable in 2 dogs; and had undergone prominent necrosis in 1. In the dog where fragments were observed, they were often surrounded by intense mononuclear cell infiltrate. Prominent arteriolar necrosis was evident in all sections. In group 4 (related allografts, CyA at 25 mg/kg/day), all the spleens had severe widespread fibrosis, and prominent arteriolar necrosis. Pancreatic fragments were seen in only 2 dogs. Most of these fragments had undergone necrosis and were surrounded by intense mononuclear cell infiltrate. In group 6 (unrelated allografts, CyA at 40 mg/kg/day), all the spleens had severe widespread fibrosis; 3 had marked plasmacytic proliferation; 2 had moderate diffuse necrosis that also involved arterioles. Intense mononuclear cell infiltrate surrounded relatively well preserved pancreatic fragments in 2 dogs; beta cells could not be identified.

Table 1. Metabolic data of groups 2 and 5 following transplantation. All subjects in these groups survived the maximal 60 day length of the study (means ± SE).

Group	Treatment	Test Subjects	Days Until Euglycemia Achieved	Maintained Euglycemia Until Day 60	K Values At 3 weeks	K Values At 8 weeks	Final Plasma Glucose (mg/dl) Before Splenectomy (Day 60)
2	Pancreatectomy + autotransplant	A, B, C	5.0 ± 2.0	Yes	1.6 ± 0.2	1.8 ± 0.3	97 ± 6.5
		D	3	No[a]	1.3	1.0	148
5	Pancreatectomy + allograft (related) + cyA (40mg/Kg/day)	A, B	6.0 ± 0.0	Yes	0.9 ± 0.4	1.7 ± 0.2	97 ± 8.0
		C	12	No[b]	0.8	0.6	214
		D	Never	-	0.8	0.3	309

[a] Developed hyperglycemia from day 35 to 49. Daily plasma glucose during this period was 202 ± 16.1 mg/dl.

[b] Became hyperglycemic on day 31 and remained so for duration of study. Daily plasma glucose during this period was 218 ± 5.1.

Table 2. Metabolic and survival data of groups 3, 4, and 6 following transplantation (means ± SE).

Group	Treatment	Test Subjects	Days Until Euglycemia Achieved	Number of Days Before Sacrificed	Mean Days Before Sacrificed	K Values At 3 weeks	Final Plasma Glucose (mg/dl)
3	Pancreatectomy + allograft (related) + No cyA	A, B, C	Never[a]	8, 26, 29	21.0 ± 6.6	0.3 ± 0.0	399 ± 26.6
4	Pancreatectomy + allograft (related) + cyA (25mg/Kg/day)	A, B, C, D	Never	20, 24, 29, 30	25.8 ± 2.3	0.4 ± 0.1	365 ± 35.6
6	Pancreatectomy + allograft (unrelated) + cyA (40mg/Kg/day)	A, B, C, D	Never	13[b], 26, 29, 39	26.8 ± 5.4	0.5 ± 0.1	336 ± 31.1

[a] Subject B had a short period of euglycemia from day 6-10.

[b] Subject A was sacrificed on day 13 due to development of severe acute hemorrhagic enteritis. Parvovirus infection was suspected.

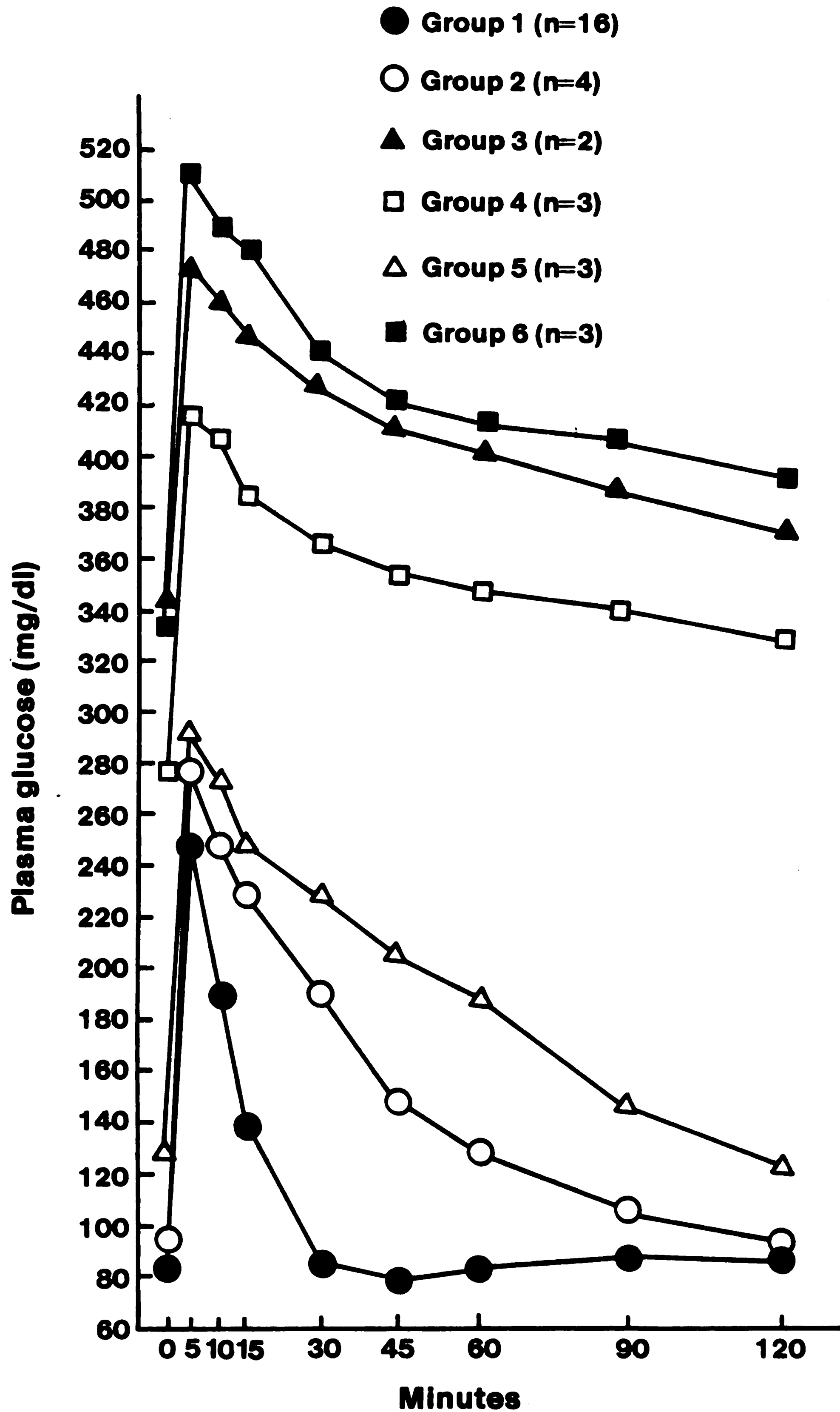

Figure 1. Mean plasma glucose values during IVGTTs 3 weeks after transplantation. Comparison is made to normal dogs (Group 1).

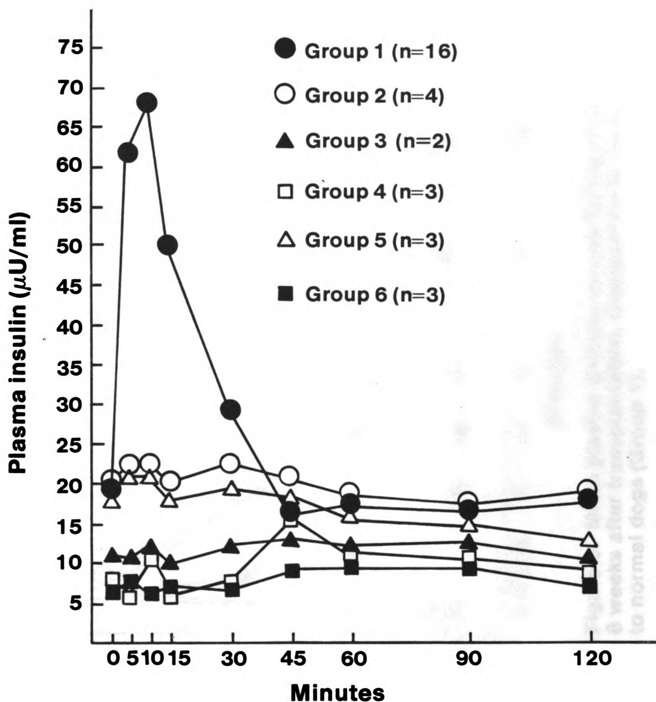

Figure 2. Mean plasma insulin levels during IVGTTs 3 weeks after transplantation. Comparison is made to normal dogs (Group 1).

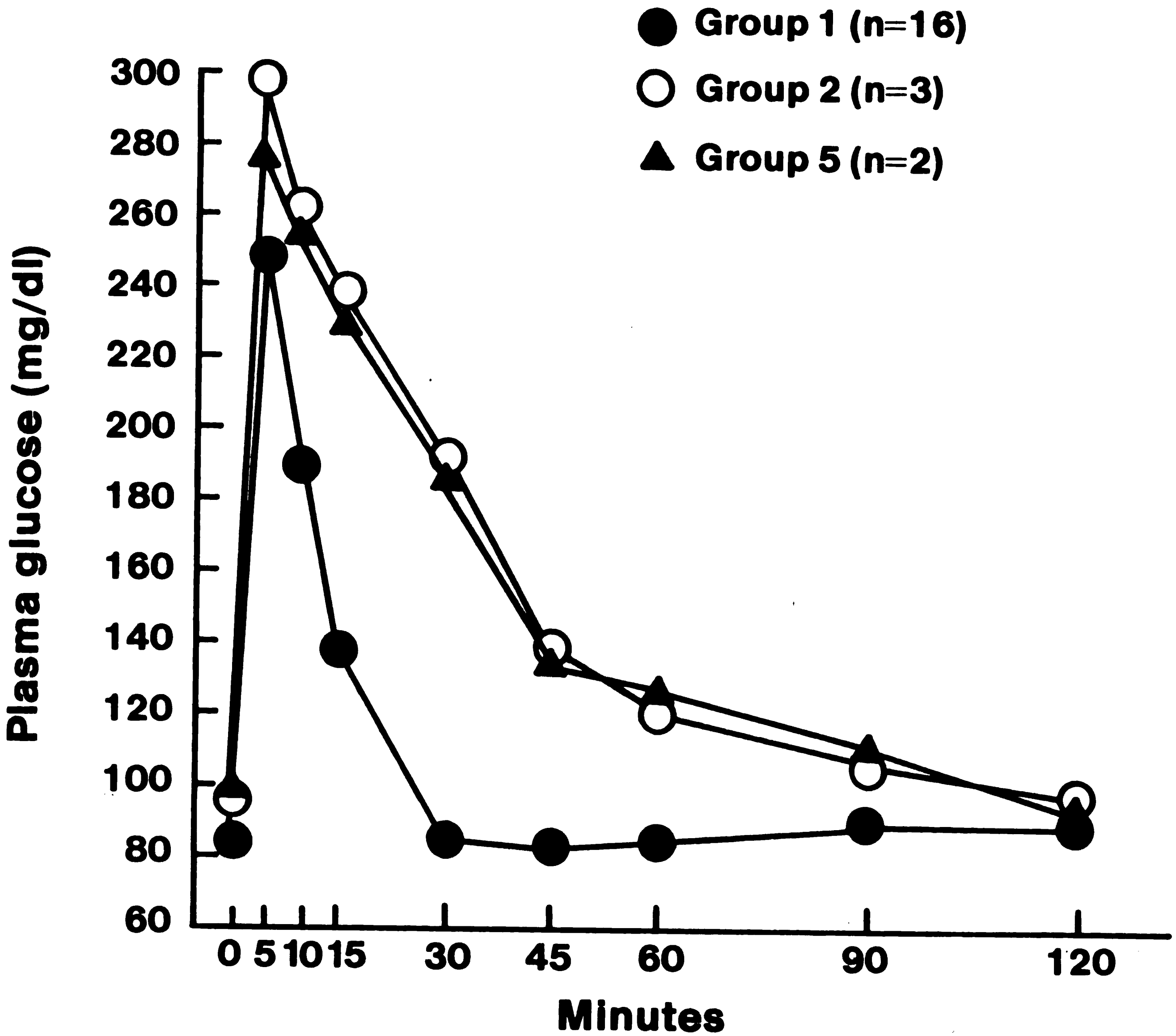

Figure 3. Mean plasma glucose values during IVGTTs 8 weeks after transplantation. Comparison is made to normal dogs (Group 1).

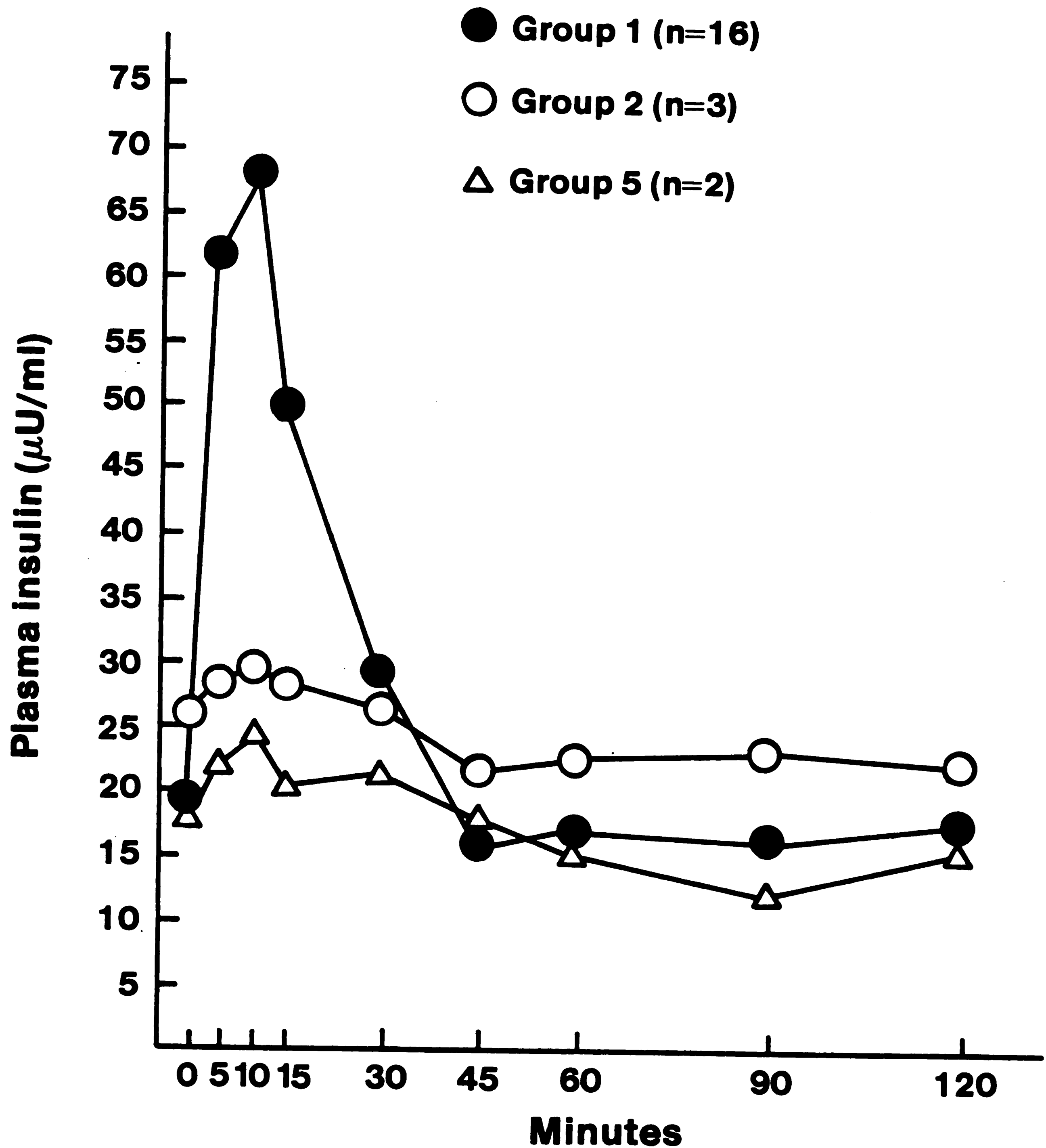

Figure 4. Mean plasma insulin levels during IVGTTs 8 weeks after transplantation. Comparison is made to normal dogs (Group 1).

SUMMARY AND CONCLUSIONS

The results of autologous transplants confirm that diabetes produced in dogs by total pancreatectomy can be ameliorated by intrasplenic dispersed pancreatic fragments. After glucose loading, however, glucose decay and insulin peaks were of a lesser magnitude than those observed in normal dogs. These findings are similar to those of other investigators (3, 12).

Intrasplenic dispersed pancreatic fragments obtained from histocompatible related donors were ineffective in ameliorating diabetes when no immunosuppressive agent was administered, nor when CyA was administered at a dose of 25 mg/kg/day.

Diabetes in dogs produced by pancreatectomy was ameliorated, however, by intrasplenic dispersed pancreatic fragments obtained from histocompatible related donors when CyA was administered at a dose of 40 mg/kg/day. Furthermore, glucose decay and insulin peaks after glucose loading were not different from those of dogs receiving autologous tissue (at 8 weeks).

Cyclosporin A administered at a dose of 40 mg/kg/day was ineffective when administered to dogs receiving tissue from unrelated histocompatible donors. This finding emphasizes the limitations of DLA and MLC as a means of determining histocompatibility and underscores the importance of minor histoincompatibility.

The dose response curve evident from differences between groups of related histocompatible dogs treated with the 2 doses (25 mg/kg/day and 40 mg/kg/day) of CyA raises the question as to whether CyA at a dose greater than 40 mg/kg/day would be effective and tolerated if used in dogs receiving tissue from unrelated histocompatible donors. Animals in this study tolerated the drug well, and there was no clinical, biochemical, or histologic evidence of toxicity.

Recent studies in rodents have shown that the antigenicity of isolated islets may be considerably reduced by tissue culture before transplantation (80-83, 85, 86); reduced to magnitude where they are less immunogenic than other tissues. It has been demonstrated that following culture at low temperature (24°C), isolated adult rat islets can be transplanted across major histocompatibility barriers, and even xenografted in mice; in each case animals were nonimmunosuppressed or received only a single injection of antilymphocyte immunoglobulin (81-83, 85, 86). This remarkable phenomena may be explained by the preferential dissolution in culture of "stimulator" cells (passenger leukocytes, macrophages, endothelial cells) implicated in allograft rejection response.

Within the past year, Lacy and co-workers have developed an improved method for specific isolation of islets from the compact pancreas of bovine and swine (84). If this recently developed isolation technique can be adapted to the dog, and followed with islet tissue culture, it may be possible to successfully transplant insulin producing tissue between unrelated, histocompatible dogs using CyA at a dose of 40 mg/kg/day or less.

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VITAE

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The author was born in Fort Belvoir, Virginia on April 29, 1954, to Neil Louis Williams, D.V.M. and Florence Hendley Williams. The first 8 years of his life were spent mainly as a resident of Gainesville, Florida, after which time his family moved to Atlanta, Georgia. His elementary and secondary education culminated with graduation from Henry Grady High School (Atlanta, Georgia) in 1972. In the fall of 1972, he began college study at Tuskegee Institute (Tuskegee Institute, Alabama), "the pride of the swift growing South". His studies at Tuskegee Institute yielded a B.S. degree in Animal and Poultry Science in 1976, and culminated with the D.V.M. (Doctor of Veterinary Medicine) degree in 1978. He began graduate study in pursuit of the Ph.D. degree in Veterinary Pathology at Michigan State University in the summer of 1978. During the course of his graduate study he was the recipient of a 3-year National Institutes of Health (N.I.H.) National Research Service Award in 1980, and was also a recipient of a Charles L. Davis Scholarship Award for Veterinary Pathology in 1982.

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