ISLET XENOTRANSPLANTATION AND XENO-ANTIGENICITY

STUDIES IN A PRECLINICAL MODEL

PLEUNIE ROOD

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Islet Xenotransplantation and Xeno-Antigenicity

Studies in a preclinical model

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studies in een preklinisch model

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Introduction and outline

In the unfortunate event of islet beta cell failure, the unequivocal patho-physiological marker of type 1 diabetes, insulin treatment is the only life-saving therapy. There is general agreement, however, that even when insulin treatment is well tolerated and carried out in a diligent way, metabolic derangements and the feared long-term complications still occur (1-3).

Beta cell replacement therapy, achieved by transplantation of whole pancreas or isolated islets of Langerhans, has emerged as the logical alternative to insulin injections. Transplantation of the whole pancreas is a complex procedure that can lead to good and long term metabolic control. However, transplantation is not devoid of complications, mainly secondary to surgery and heavy immunosuppressive therapy (4). Therefore, pancreas transplantation is recommended only to selected groups of patients, especially if performed as a solitary (not combined to a kidney graft) pancreas transplantation (5, 6).

ISLET CELL TRANSPLANTATION

Since the late 1980's, the feasibility of isolating and purifying human islets from pancreatic organs of deceased donors raised hope that purified pancreatic islet cells, rather than an entire gland, could cure diabetes (7). However, the supply of human donor pancreases from deceased donors as sources of islets is insufficient. Even in the most successful trials, multiple transplantations are necessary to obtain (temporary) normalization of hyperglycemia in the recipients (8-10). Despite significant efforts to improve the yield of isolated islets, to choose better culture conditions and preserve higher numbers of isolated islets for clinical transplantation, it became evident that the number of deceased donors available each year for islets would never be able to meet the demand.

The paucity of organ donors is one of the limiting factors to success and long-term function of islet transplants. It is now known that the quality of the isolated islets is strongly affected by the medical conditions of the organ donor, the cause of death and pre-existing diseases (11). Islet quality is moreover influenced by the events that characterize the procedure of organ donation and harvesting; instances that, together, lead to or contribute to exacerbation of tissue ischemic damage, thus deterioration of the organs (12). There is no 'healthy' organ donor after severe events such as strokes, or motorcycle accidents causing intracranial hemorrhage, the most common causes of irreversible brain damage. Brain death itself can be the cause, among many effects, of metabolic imbalance, including abnormal, typically very high, blood glucose levels (13). Combined, these factors increase the risk of worsening the quality of the islets.

There are additional logistic problems, such as the fact that the hospitals where organ retrieval takes place, are often far away from the centers that prepare the islets. Islet isolation is a rather long (6 to 8 hours) and not trivial procedure, requiring well-trained teams available

on a 24/7 basis to meet the time constrains of the organ donation programs. Despite organizational coordination efforts between the procurement agencies and the isolation centers, prolonged storage time of the pancreas, an obvious negative condition, is practically unavoidable. Marked improvements in the storage procedures and selection of preservatives have contributed to increasing the survival of the tissue (14-16) but, in practice, 8 to 12 hours on average separate the time of harvesting from the time of processing.

The ultimate therapeutic strategy for the cure of diabetes, envisions the ability to 'generate' beta cells in the laboratory from immature human cell sources that would be capable of proliferating and differentiating into insulin producing cells, in a well controlled process. This definitive strategy would not only treat patients, but also eliminate the problem of the poor availability and quality of human donor pancreases (17, 18). Ongoing studies, still in their infancy, will hopefully offer tangible results, meaning protocols transferable to clinical use. Meanwhile, we have the possibility to explore a readily-available, fully-differentiated and functional product existing in nature, namely animal islets.

XENOTRANSPLANTATION

The concept of turning to animal tissue for transplantation in humans is well-established. However, pioneer experiments of xenotransplantation using vascularized organs, as well as isolated cells, made it clear that humans and large mammals, in spite of their physiological similarities, exhibit stringent tissue incompatibilities. Mainly because of recent improvements in genetic engineering techniques, and a better understanding of the molecular mechanisms that regulate the immune responses across species, xenotransplantation has now regained scientific consideration (19).

For various reasons the pig is considered the preferred source of pancreatic xeno-islets. Pig insulin, which differs from the human type by only one amino acid, is active and well tolerated in humans. For years prior to the production of human recombinant insulin, patients were successfully treated with injections of insulin extracted from swine. Evidence that pig islets can be used for transplantation into humans was originally provided, in particular, by studies conducted during the 1980's in Sweden (20). Moreover, pig islet cells can be successfully isolated and purified from adult pigs with a method that is similar to the one used for human islets, with the advantage that the total islet mass isolated from adult pigs is larger than in humans (21). Advantages of using pigs as a supply of islet cells for transplantation are, at least in theory, numerous. Besides the benefit of a basically unlimited tissue supply, we anticipate a higher quality of the donor organs, as a direct consequence of the absence of brain death. We could, for instance, plan elective organ harvesting, therefore minimizing cold ischemia and consequently improving islet yields (22). Overall, the quality of the isolated islets would be higher.

What are the major obstacles to successful pig islet transplantation in humans? Xenotransplantation, like to a lesser extent in allotransplantation, entails the problem of rejection. Rejection of the graft (i.e., the implanted cells) across species is more aggressive than between individuals of the same species. Generalized suppression of the immune system in the recipients by pharmacological agents, while not optimal, is an effective way to control rejection. Administration of immunosuppressants is a necessary, but not fully benign, procedure that requires life-long use of drugs that restrain the immune responses. Such immunosuppressive protocols, at the doses and in the combinations currently adopted in clinical trials, would not be sufficient to prevent the prompt and massive rejection that characterizes xenotransplantation (20). One of the main reasons why tissues across species are readily destroyed is the reactivity mediated by 'preformed' or 'natural' antibodies in the recipients (humans and Old World monkeys) against epitopes on pig donor cells (23).

α1,3 GALACTOSYL TRANSFERASE KNOCKOUT (GT-KO) PIGS

What are the preformed antibodies? Pig tissue expresses the carbohydrate galactoseα1,3galactose (Gal) (24). Humans and monkeys have an impaired gene that encodes the enzyme α1,3- galactosyltransferase (GT), which mediates the expression of Gal on the cell membrane. Accordingly, they do not express such a carbohydrate. Consequently, humans promptly generate an antibody-mediated response when exposed to tissues from Gal-positive animals and microorganisms. These anti-Gal antibodies are the most important preformed antibodies in regard to xenotransplantation. They are able to mount a rapid, deleterious reaction that is the major cause of xenograft loss in the first minutes to hours after transplantation. This is known as hyperacute rejection (25). To avoid the hyperacute rejection of tissue from Gal-positive pigs (i.e., WT pigs), the possibility of generating pigs genetically deprived of the activity of the GT-enzyme was considered.

The first step was the cloning of the GT gene of the pig, an assignment completed at the end of the year 2000. Based on the gathered DNA sequence information, it became clear that an essential region of the GT gene was located in exon 9, and that the inactivation of the gene, once the coding sequence was interrupted, was not fatal for the embryo. Thus, genomic sequences encompassing the beginning of exon 9 were used to construct knockout (KO) vectors aimed at the targeted insertion of a selectable marker gene, and at the inactivation of the GT gene in pig fetal fibroblasts. Single, or heterozygous, GT gene-knockout cells were isolated and used as donors for somatic cell nuclear transfer (cloning) into fecundated eggs to produce heterozygous GT gene-knockout pigs (26). University of Pittsburgh scientists collaborated with the Virginia branch of the Scottish company, PPL Therapeutics Inc. (now Revivicor, Inc.), the same institution in Europe that was responsible for cloning the first mammal, 'Dolly' the sheep.

To produce animals useful for transplantation, it was necessary, however, to also knockout the second, yet uncompromised, allele of the gene that was responsible for the expression of Gal on the heterozygous GT-knockout pig cells. By developing a new technical approach, different from the one used for knocking-out the first allele, it was then possible to produce the first cloned pigs homozygous for α1,3 galactosyl transferase gene-knockout (GT-KO) (27).

The availability of pigs with tissue that lack the most important rejection-inducible determinant, and that is presumably more compatible for transplantation in humans, fostered an intensive period of experiments. Clinical trials cannot be started without appropriate validation of results from rodent studies in preclinical studies, meaning using nonhuman primates. In the past, several groups have studied transplantation of (unmodified) porcine islets into nonhuman primates, usually with unsatisfactory outcome (28). Clearly, satisfactory and safe preclinical studies are mandatory before proceeding to clinical trials.

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OUTLINE OF THIS DISSERTATION

As outlined above, a shortage of human donor organs is the major limiting factor for clinical islet allotransplantation. Xenotransplantation, using the pig as the source of islets, is considered a potential solution to this problem. Since the development of pigs homozygous for α1,3-galactosyltransferase gene-knockout (GT-KO), renewed interest exists in the possibility of pig-to-primate xenotransplantation. This thesis involves several aspects of islet xenotransplantation in a pig-to-nonhuman primate model and of the xeno-antigenicity of donor pig tissue.

The first aim was to review the current state of pig-to-nonhuman primate islet xenotransplantation documented at the time the personal studies reported in this dissertation were initiated (**Chapter 2**).

The second aim was to focus attention on the nature of xeno-antibodies in primate sera directed against pig cells. Anti-pig antibodies are formed against galactoseα1,3galactose (Gal) and nonGal epitopes on pig cells. As described above, anti-Gal antibodies are responsible for hyperacute rejection of pig grafts. The availability of GT-KO pigs enabled us to study the presence of anti-nonGal antibodies in baboons, cynomolgus monkeys and humans. Different aspects were studied. The binding and cytotoxicity of preformed antibodies to nonGal antigens were documented in baboons, monkeys, and humans (**Chapters 3-5**). Furthermore, we investigated the development of an elicited nonGal antibody response after GT-KO pig organ transplantation in baboons (**Chapter 3**). Additionally, the potential difference in anti-nonGal antibody levels in allosensitized compared to nonsensitized humans was determined (**Chapter 4**). Differences in anti-nonGal antibody levels between different species (i.e., baboons, monkeys and humans) were determined (**Chapter 5**). We studied specifically the development of anti-nonGal antibodies in infant humans and infant baboons. The possible implications for clinical xenotransplantation were addressed (**Chapter 6**).

The next aim was to study porcine islet xenotransplantation in vivo in a nonhuman primate model. The first step was to establish a reproducible and safe model for the induction of diabetes in cynomolgus monkeys (**Chapter 7**). We were then able to study the intraportal transplantation of WT and GT-KO pig islets into diabetic cynomolgus monkeys. The rapid and massive loss of islets shortly after intraportal transplantation (described earlier as the instant blood-mediated inflammatory reaction [IBMIR] by the Uppsala group) was confirmed, and an attempt was made to overcome this destruction by investigating different ways to reduce early graft loss (**Chapter 8**).

The final aim was to evaluate future perspectives. The current position of clinical islet xenotransplantation was discussed (**Chapter 9**), and a possible alternative to islet xenotransplantation, i.e., islet cell regeneration, was described (**Chapter 10**).

Chapter 2
Pig-to-nonhuman primate islet xenotransplantation: A review of current problems

P.P.M. Rood, L.H. Buhler, R. Bottino, M. Trucco, D.K.C. Cooper. Cell Transplantation, 2006;15(2):89-104

ABSTRACT

Islet allotransplantation has been shown to have potential as a treatment for type 1 diabetic patients. Xenotransplantation, using the pig as a donor, offers the possibility of an unlimited number of islets. This comprehensive review focuses on experience obtained in pig-to-nonhuman primate models, particularly with regard to the different types of islets (fetal, neonatal, adult) and isolation procedures used, and the methods to determine islet viability. The advantages and disadvantages of the methods to induce diabetes (pancreatectomy, streptozotocin) are discussed. Experience in pig-to-nonhuman primate islet transplantation studies is reviewed, including discussion of the possible mechanisms of rejection and the immunosuppressive regimens used. The research carried out to date has led to workable animal models to study islet xenotransplantation, but several questions regarding methodology remain unanswered, and details of these practicalities require to be adequately addressed. The encouraging porcine islet survival reported recently provides an indicator for future immunosuppressive regimens.

INTRODUCTION

The relative success of the Edmonton protocol has proven that clinical islet allotransplantation is possible with a steroid-free immunosuppressive regimen (1). Unfortunately, the problem of shortage of human donor islets is the major limiting factor for clinical allotransplantation. Xenotransplantation, using the pig as the source of islets, is a potential solution to this problem (2). Many groups have studied islet xenotransplantation in different animal models and in vitro. Although it is clear that there are still immunological, and possibly other, barriers that have to be overcome before islet xenotransplantation will become clinically applicable, progress is taking place. We have therefore reviewed work reported in pig-to-nonhuman primate models.

Reviews of the general field of islet xenotransplantation have previously been published by MacKenzie et al. and Berney et al. (3, 4), and Mandel reviewed the field of fetal islet xenotransplantation (5). We here focus on preclinical trials in nonhuman primates, including discussion of the various types of pig islets that have been investigated (e.g., fetal, neonatal, adult), the islet isolation procedures used, the methods used to induce diabetes in nonhuman primates, the mechanisms by which transplanted pig islets are believed to be rejected, and the various immunosuppressive regimens that have been tested.

SOURCE, ISOLATION, AND PREPARATION OF PORCINE ISLETS

Strain

Heiser et al. studied the influence of porcine strain on islet yields, comparing young or adult crossbreeds, German Landrace, Pietrain, and Munich minipigs; the best and most reproducible yields were achieved from Pietrain pigs (6). Toso et al. shared the conclusion that Pietrain pigs seem to be most suitable (7). However, data on significant strain differences are not conclusive.

Fetal islets

Because of the relative lack of exocrine tissue in the fetal pancreas, the isolation of fetal islets requires less complex isolation techniques and is therefore easier (5). Korsgren et al. initially advised the use of fetuses 'as old as possible', although pancreases of fetuses of late gestational age (± day 112-113) were found more difficult to digest (8). More recently, fetuses of 70-86 days of gestation have been used as their islets are believed to have better survival potential (9, 10). During the isolation process, the pancreatic tissue is simply mixed with exogenous digesting enzymes, the mixture is incubated and vigorously stirred for 20 to 30 minutes, after which the digestion is stopped. This method does not require purification prior to islet culture (8, 10).

Based on results from experiments in mice, Mandel estimated that, considering its growth potential, one pig fetus would provide enough islets for one human recipient if rejection did not reduce the number (5). Unfortunately, to date, rejection does play a role, and the optimum number of islets to reverse diabetes in primates remains to be determined.

Fetal islets are more resistant to ischemic injury after pancreas procurement, probably due to the relative lack of exocrine tissue retained within fetal islet cell clusters, and the characteristics of fetal tissue itself (5). Precursor cells, that are located in the pancreatic ducts and probably in immature islet cells, allow proliferation after transplantation (11).

Ironically, the major disadvantage of fetal islets is their immaturity, which is associated with delayed function of the islets after transplantation (8). It may take over two months, sometimes up to 6 months before normoglycemia is restored (12, 13); during this period, therefore, there is a negligible or poor insulin response to glucose. A second disadvantage is the expression of Galactoseα1,3Galactose (Gal) on the surface of fetal pig islets, rendering wild-type porcine fetal islets more susceptible to rejection than adult islets, which express no or little Gal (discussed below) (14-22).

In view of the relatively simple technique of fetal islet isolation, and the relative low costs involved, fetal islets are still popular for rodent studies (Figure 1A), but an increasing ability

to achieve good adult islet isolation has stimulated a move away from fetal islets in primate studies.

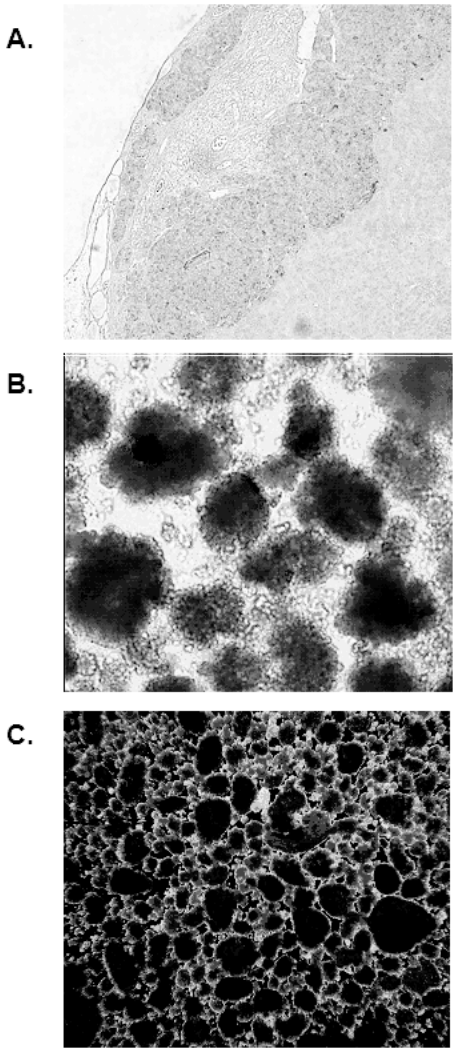

Figure 1:
(A) Fetal pig ICC, transplanted under the kidney capsule of a mouse, stained with anti-insulin antibodies, **(B)** Neonatal pig ICC, after isolation, stained in vitro with dithizone (dithizone stains red for insulin-containing cells), **(C)** Adult pig islets, after purification, stained in vitro with dithizone.

Neonatal islets

Several groups have isolated islets from pigs within the first postnatal month, usually within the first days (22-25) (Figure 1B). Although insulin secretion is delayed after transplantation, the period is generally shorter than after fetal islet transplantation (26-28).

Korbutt et al. (23) established an isolation technique for neonatal islets. Briefly, pancreatic tissue from 1 to 3 day old piglets was digested enzymatically using collagenase-type enzymes for 16-18 minutes, and the islets were cultured for 9 days, during which albumin, IBMX (3-Isobutyl-1-methylxanthine, a phosphodiesterase inhibitor and insulin secretagogue), glucose, and nicotinamide (but no serum) were added. This resulted in yields of 50,000 neonatal islet cell clusters (ICC) per pancreas, compared to an average of 10,000 ICC following mid-gestational fetal islet isolation. After transplanting the neonatal islets into diabetic nude mice, there is however a delay of 6 to 10 weeks before diabetes can be reversed (22).

Juang et al. (24) compared the characteristics of islets derived from 1 to 3 day-old piglets with 1 month-old piglets; their response to glucose stimulation in vitro during incubation, as well as the beta cell mass and function after islet harvesting, were similar.

Adult islets

Compared to humans, the peri-insular matrix, 'the islet capsule', in adult pigs is poorly developed, making the islets susceptible to fragmentation and loss during the isolation process. Islets from young adult pigs are particularly fragile. According to some reports, young pigs do not have an islet capsule at all (29); others report that young pig islets have lower resistance to the digesting enzyme, collagenase (7).

Generally, retired breeders (i.e., sows that have undergone multiple pregnancies) are preferred as donors of adult porcine islets (30) (Figure 1C). In humans, pregnancy induces increases in the beta cell mass, the beta cell sensitivity to glucose, and its production of insulin (31). It is these changes that are assumed to result in better islet yields from retired breeders, even though it has been reported in rats that the beta cell mass regresses to normal (pre-pregnancy) state within 10 days post-partum (32).

To our knowledge, there are no studies comparing the yields of islets from adult male pigs of comparable age with those from female retired breeders. In general, 2-3 year-old pigs are preferred over younger pigs (i.e. <14 months) because of the relative structural stability (or reduced fragility) of the islets. However, successful islet isolation from younger pigs is possible, and reasonably good survival of islets has been achieved (30, 33).

Other variables influencing islet yields

Retained blood in the pancreas after pancreatectomy results in reduced islet yield, possibly through inhibition of the collagenolytic activities of the digestive enzymes (34). Extensive flushing before, during, and directly after pancreatectomy is necessary. The diet of the pig can also influence the yield, possibly through stimulation of intrinsic enzymes that are mainly responsible for autolysis after pancreas procurement. Nutritional substrates may account for lower yields (7); islet isolation from pigs following a diet enriched with soybean oil gave better yields than those from pigs following a coconut oil regimen (35).

The effect of warm and cold ischemia times on islet yields

The period of warm ischemia must be minimal, since, during this period, autolysis of the pancreas is induced by proteolytic and lipolytic enzymes (7, 36, 37). When the warm ischemia time approached 20 minutes, losses in the number of islets after isolation of up to 70% were seen (38).

It seems inadvisable to use pig pancreases with a cold ischemia time (i.e. the period that elapses between putting the pancreas in ice after procurement and the start of the isolation process) of >12 hours (37). In concordance with the results achieved in human islet isolation (39), Brandhorst et al. demonstrated that the two-layer method of isolation, using perfluorocarbon as an oxygen carrier, may enable an extension of the acceptable period of cold ischemia; improved numbers of pig islets were isolated after 7 hours of cold preservation by this technique (40). Recently, their group demonstrated that when perfluorocarbon was used in a simplified one-layer method, successful pig pancreas preservation was also achieved (41).

The intraductal injection with collagenase-containing solutions before storing the pancreas contributes to higher yields (7), and injection of collagenase both intraductally and directly into the pancreatic tissue is preferred over intraductal injection alone (7). However, using the two-layer method, Brandhorst et al. showed that enzyme administration early after pancreas procurement decreased islet graft function if the period of cold ischemia had been prolonged (40).

Adult pig pancreatectomy

Surgical techniques for pancreatectomy in the pig have been described by Morel et al. (42). The pig pancreas has an anatomical localization and orientation similar to that of the human pancreas (42), although variability of blood supply has been reported (43). To avoid leakage of digesting enzymes during subsequent digestion, it is greatly preferred that the pancreas is excised with an intact pancreatic capsule.

Preparation of adult islets:

Digestion

Continuous exposure to digesting enzymes is of importance, but this must not be too prolonged or injury to the islet capsule ensues, damaging the islets themselves. The optimum end-point of digestion is difficult to determine.

Since its introduction by Ricordi et al. in 1990, most groups use semi-automatic isolation whereby the islets are shaken in a digestion chamber, and the temperature is controlled between 34-37 ºC (36). O'Neil et al. described static incubation in a 37ºC water bath for 25-65 min; this has proved a relatively simple method, providing good results (30).

The digesting enzymes usually include collagenase, which consists of up to 6 enzymes and is naturally produced by Clostridium histolyticum. 'Liberase' is a purified collagenase that has the advantage of less inter-lot variation compared to collagenase (33, 37). Brandhorst et al. have shown higher yields and less fragmentation utilizing Liberase HI compared to collagenase (44); more recently, Liberase PI has been introduced for pig islet isolation (45). Hanks' balanced salt solution or University of Wisconsin solution are the most widely used carriers for the digesting enzymes (7). The pH of the solution isolation medium influences the yield, with a pH >7.3 resulting in higher yields (6).

Purification

Digestion is followed by purification of the islets. As it is immunogenic and inflammatory, the remaining exocrine tissue is removed. The accepted method is density-gradient centrifugation, since islets are slightly less dense than exocrine tissue. Ficoll, Euroficoll, Ficoll diatrizoic acid, hyperosmolar bovine serum albumin are employed in this process at 4ºC (46). Purity levels of 80-90% are usually obtained (7). Optional methods employed are immunomagnetic cell separation, whereby immunomagnetically-labeled digested pancreatic tissue is separated in a magnetic field (47), and fluorescence activated cell sorting (48-50).

Culture of islets

Fetal and neonatal islets have to be cultured to allow them to reaggregate into islet-like cell clusters, which takes at least 4 to 5 days (8, 51, 52). Adult pig islets fragment more easily (53), which is a disadvantage to the culture of these islets, as are the risks of infection and loss of viability. However, culture allows for some pre-transplantation immunomodulation (54), as well as further purification (55); culture is also a form of storage. There is no agreement on the optimum period of time for adult islet culture, which has been continued for 24 hours to several weeks. There is also no generally-agreed ideal islet culture medium; RPMI 1640 and CMRL 1066, supplemented with serum-derived proteins, are the most frequently used. Good oxygenation during culture is essential, and the preferred temperature is 22-24˚C (37).

Cryopreservation provides an alternative to culture as a means of storage, but islet viability is always reduced, and subsequent function is also impaired (56). Storage of pig islets may be largely unnecessary, as a donor pig will be available on demand.

Viability and functional assays

Islet viability is strongly influenced by warm and cold ischemia times, the isolation process, and the period of storage (whether this be cryo or culture), but it is difficult to evaluate the exact influence of each (7, 29, 37). Viability can be determined by electron microscopy (57), double fluorescent staining (58), fluorometric membrane integrity (59, 60), or by mitochondrial function, measured by oxygen consumption (48, 61).

Functional assays include in vitro glucose-stimulated insulin release, and in vivo function is determined by transplanting the islets into small animals, such as diabetic immunodeficient (NODscid) mice (7, 30, 37), which can be expected to become normoglycemic within days (62, 63). An intraperitoneal glucose tolerance test may provide further information (64).

THE INDUCTION OF DIABETES IN NONHUMAN PRIMATES

It is generally preferred to render the recipient diabetic to allow assessment of the capability of the graft to induce normoglycemia, even though measurement of pig C-peptide in a nondiabetic, nonhuman primate recipient provides an indication of the viability of the transplanted islets.

Spontaneous diabetes

Spontaneous diabetes has been reported in several strains of monkey, including cynomolgus and rhesus monkeys (65-67), although their availability is limited, and it is absolutely necessary to verify pure type I diabetes.

Surgical pancreatectomy

Pancreatectomy is not without surgical morbidity and has the disadvantage of loss of exocrine pancreatic function (65, 68). There is the potential for continuing function or regeneration of beta cells if the pancreatectomy has not been total.

Chemical induction

Increasingly, streptozotocin (STZ), derived from the bacteria Streptomyces achromogenes, is being administered intravenously to the potential recipient to induce diabetes. Besides antimicrobial and anti-tumor activities, STZ is diabetogenic through oxidative stress resulting in selective death of the beta cells (69). Nephrotoxicity and hepatotoxicity are disadvantages of STZ (70-73). In rodents, regeneration of beta cells has been observed, resulting from intra-islet progenitor cells, that are possibly not affected by STZ treatment (74-77). However, STZ-induced diabetic monkeys remained diabetic for at least one year, with no significant primate C-peptide release after glucose stimulation, and pancreas histology that demonstrated only a few insulin-staining cells (70).

STZ administration to induce diabetes has several potential variables (Table 1). The first relates to the optimal dose of STZ in nonhuman primates. Pitkin et al. and Litwak et al. showed that a single dose of 30mg/kg is not sufficient to induce complete diabetes (78, 79). Larger doses of 100-150mg/kg were found sufficient (70, 72, 80), but were associated with more side effects and complications (70-73, 80). In contrast, Koulmanda et al. reported that 55mg/kg was sufficient to consistently induce diabetes in cynomolgus monkeys (70), but others were less successful (71, 78, 81). Since STZ has a half-life of only 10 min (70), it

Table 1:
Selected experience in induction of diabetes by streptozotocin in nonhuman primates

author		(n, when stated)	(when stated)	(mg/kg)	(C-pep in ng/mL)
Wijkstrom	(80)	Cyno (26) Rhesus (21)	3.3 ± 1.2 kg 4.6 ± 1.1 kg	133 ± 21 143 ± 17	46/53 diabetic. Complications were dose dependent; death 5/53, renal toxicity 8/53, hepatotoxicity 18/53. Concluded safe and effective dose: 1250 mg/m^2
Koulmanda	(70)	Cyno (20) Cyno (4)	3.1-6 kg/ 3-6 years	55 100	All groups diabetic (C-pep <0.6)
Contreras	(86)	Rhesus	3.0-3.8 kg	140	All hyperglycemic (C-pep< 0.6)
Gaur	(73)	Pigtailed macaques (15)	< 15 kg	10-40, 1-3 doses	13/15 hyperglycemic after one injection (25 or 40 mg/kg). Multiple injections affected kidney and liver functions.
Thieriault	(72)	Cyno (11)	1 - 2 kg/ 6-8 months	150	All monkeys hyperglycemic (C-pep < 0.9)
Litwak	(79)	Cyno (8)	4.9 ± 0.4 kg/ 6.2 ± 0.8 years	30, 1-3 doses	3/8 hyperglycemic after 1 dose 1/8 glucose intolerant after 1 dose 3/8 hyperglycemic after 2 doses 1/8 hyperglycemic after 3 doses
Stegall	(71)	Cyno (>20)	2.5 - 4.4 kg	50	80 % diabetic after 1 dose
Takimoto	(81)	Rhesus (22)	8.45 ± 1.43 years 8.3 ± 2.47 years 6.01 ± 0.94 years	45-55	10/22 diabetic within 5 days (C-pep < 0.06) 4/22 non insulin-dependent 8/22 non diabetic
Jones	(125)	Rhesus (20)	5.2 ± 0.2 kg 5.5 ± 0.4 kg	30-55 30-55	11/20 insulin-dependent 9/20 impaired glucose tolerance
Pitkin	(78)	Rhesus (3) Rhesus (7) Rhesus (2)	3.8 - 6.2 kg	30 45 60	None diabetic 5/7 hyperglycemic, 2 glucose intolerant All hyperglycemic

Abbreviations: C-pep= C-peptide; Cyno= Cynomolgus monkey; Rhesus= Rhesus monkey

seems reasonable to anticipate that higher dosages are necessary if it is not administered rapidly as a bolus. A significant loss of efficacy will ensue if the STZ is infused slowly over a period of minutes. Wijkstrom et al. suggested the dose should be based on body surface

area (1250mg/m^2), rather than body weight (80). The islets of younger nonhuman primates may be more difficult to destroy, and this may account for the varying STZ dosages required to induce diabetes (70, 81). There is a reported correlation between the dose of STZ and the development of renal and hepatic damage, as documented by an increase in blood urea nitrogen, creatinine and/or hepatic enzymes, respectively, or by histology (70-73). To diminish or prevent kidney damage, hydration both before and after STZ administration is recommended (70-72, 82).

Zanosar (Sicor Pharmaceuticals, Inc.) is a form of STZ that is used clinically in patients with metastatic islet cell carcinoma and may have fewer side effects than other preparations of STZ, perhaps because of greater purity and less variability. Zanosar has consistently induced diabetes in nonhuman primates at a single dose of 150mg/kg administered rapidly, without significant side effects (S. Deng, personal communication). It would seem that a dose of >100mg/kg or 1250mg/m^2, administered as a rapid bolus, has the potential to induce diabetes consistently.

Whereas some investigators begin immunosuppressive therapy for islet transplantation one week after STZ administration, others wait months to confirm the diabetic state. Administration of cyclosporine within 1 to 2 weeks after STZ resulted in renal failure (83). Our own limited experience suggests that immunosuppressive therapy within 2 weeks of STZ administration may aggravate an STZ-associated glomuleronephropathy.

Alloxan has been used to chemically induce a diabetic state, mainly in rodents. Although nonhuman primates have been reported to become diabetic after alloxan administration (65), it is generally assumed that alloxan works selectively on rodent beta cells, and is therefore not a potent inducer of diabetes in nonhuman primates (84, 85).

Confirmation of the induction of diabetes

Hyperglycemia has been used to indicate induction of diabetes (71, 78), but C-peptide levels are generally measured to confirm it (Table 2). Primate C-peptide levels of <0.9ng/mL were considered sufficient to indicate a diabetic state by Thieriault et al., whereas others required levels <0.6ng/mL (70, 81, 86) or <0.4ng/mL, as well as an absence (<0.1ng/mL) of C-peptide response to arginine challenge (80). Only meticulous recipient total pancreatectomy (but not STZ administration) results in a C-peptide level of zero (70).

Some form of glucose challenge is required to confirm diabetic status (37, 70-73, 78, 79, 81, 86). Arginine (70 mg/kg over 30 min) (87) stimulates both alpha and beta cell activity; the absence of C-peptide and insulin responses after arginine administration confirm diabetes (82, 88, 89). Glucagon stimulation is a clinical test that has not yet been described in the nonhuman primate setting; it is a simple, quantitative test that measures the responses of glucose, insulin, and C-peptide to 1mg glucagon in human adults (90).

Table 2: Reported C-peptide values in humans, monkeys, and pigs (ng/ml)

	Human (ref)	Monkey (ref)	Pig (ref)
Non-diabetic			
Fasting:	0.5-1.5 (126)	1.5-4.1(11)	0.21-0.63 (127)
Diabetic			
Challenged:	< 0.48 (1)	<0.5 (80)	

The effect of STZ on the beta cells can also be evaluated by histological examination of a pancreatic biopsy, but this requires a surgical procedure, and some immunostaining for insulin is always present. Whereas healthy non-diabetic cynomolgus monkeys have 10-30 insulin-positive cells per islet (70), Koulmanda et al. reported <5 insulin-positive cells per islet after STZ. Litwak et al. reported 18% insulin staining in pancreases of STZ induced diabetic monkeys, compared to 61% insulin staining in control monkeys (79).

EXPERIENCE IN EXPERIMENTAL PIG-TO-NONHUMAN PRIMATE ISLET TRANSPLANTATION

The pig-to-nonhuman primate islet transplantation experiments reported in the literature are summarized in Table 3.

Recipients for nonhuman primate experiments

Species

Macaque monkeys, especially cynomolgus monkeys, have been the major species of choice (Table 3). Macaque monkeys are generally smaller than baboons, and therefore have the advantage of requiring less expensive housing, husbandry, and drug costs; and fewer pig islets are needed to be transplanted. Baboons, though used far less often, are perhaps more hardy than macaque monkeys, possibly rendering them less susceptible to surgical and other complications.

Diabetic status

The majority of experiments have been performed in non-diabetic recipients (82, 91-96). This model allows only evaluation of porcine C-peptide production, and the immune response, including measurement of complement activation, anti-pig antibody status, and graft histology

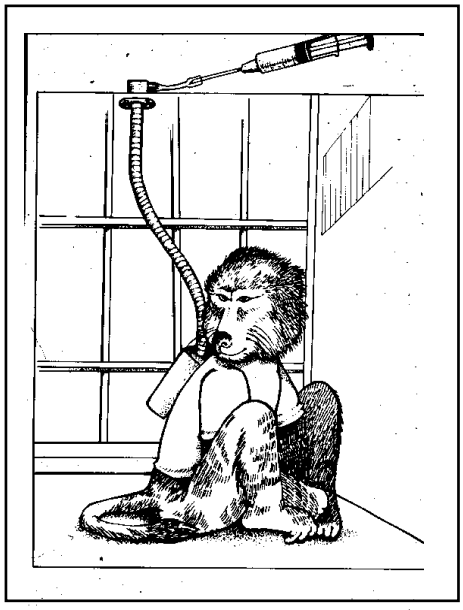

Figure 2:
Drawing showing system allowing continuous or intermittent hemodynamic monitoring, drug and fluid infusion, and blood sampling in the nonhuman primate without the need for sedation. Venous (femoral or jugular) and/or arterial (femoral or carotid) cannulae are inserted, tunneled subcutaneously to the middle of the back and brought out through a protective flexible metal tube (held in place by a jacket).

(94), but not the ability of islet grafts to normalize diabetic status. The advantage of having diabetic recipients is the ability to follow blood glucose levels after islet transplantation (66, 82, 89, 92, 94). Neither the histological presence of islets, nor the documentation of porcine C-peptide necessarily correlate with functional reversal of diabetes (9).

Tethering

Except for one study by Buhler et al. (92), and in our own unreported experience, all studies in the pig-to-nonhuman primate model have been carried out in untethered animals. Tethers carry indwelling intravascular and/or gastric catheters (Figure 2), and provide an advantage in regard to the ease with which blood samples can be obtained and fluids and drugs administered, without the need for restraining or sedating the primate (97). This is especially advantageous in diabetic monkeys that need frequent blood glucose monitoring. However, the intravascular catheters require skillful and careful handling, and the potential for introduction of a systemic infection and for hemorrhage or embolism represents a small risk.

Site of islet transplantation

The majority of studies have placed the porcine islets under the recipient's renal capsule or intraportally (Table 3), though some have been transplanted subcutaneously (93) or into the sternomastoid muscle (92). Sun et al. transplanted islets (encased in alginate capsules) intraperitoneally (66, 98).

After intraportal islet transplantation, the islets lodge in the small venules of the liver, where they produce insulin in response to portal blood glucose values. In an allograft setting, capillary vascularization of the islets has been demonstrated to occur within weeks (99). The disadvantage of this approach is that direct exposure of the islets to the blood can result in an immediate inflammatory response and complement activation ('instant blood-mediated inflammatory reaction' [IBMIR] (94)), and activation of both the humoral and cellular responses (see below) (82). Furthermore, obtaining liver biopsies that confirm the presence of viable islets, which may be widely-dispersed throughout the liver, is difficult; indeed, partial hepatectomy may be required to obtain meaningful histology (B.J. Hering, personal communication).

When placed under the renal capsule, the islets are safer from immediate destruction by the immune system, although ischemic injury can be a problem (95). The islets also need to be revascularized by the recipient, which usually takes about 10 days (100), but may take 3 to 5 weeks in the human-to-mouse model (101). Repeated biopsy, though invasive, is relatively easy. However, low or no porcine C-peptide production has been reported from subrenal capsular islets in the pig-to-nonhuman primate model (Table 3).

Porcine islets transplanted into the subcutaneous tissues showed an early inflammatory response, and only a few islets survived for more than 3 days (93). As early as 1996, Sun et al. reported survival and C-peptide production of encapsulated porcine islets transplanted into the peritoneal cavity in cynomolgus monkeys for up to 804 days, but no further studies have been reported (66).

Number of transplanted islets

The number of islets that can be transplanted intraportally is limited because of the risk of portal vein thrombosis. To reduce this risk, the islets are mixed with heparin (usually 70U/kg recipient body weight). In our own experience, this dose moderately increases the partial thromboplastin time of the recipient, but for <1 hour. The maximum number of islets reported to be successfully infused by this route (without complications) has been 32,000 IE/kg (92), although we have recently transplanted 40,000 IE/kg without problem.

There appears to be no strict limitation on the number of islets that can be transplanted subcapsularly, although the numbers have not been higher than those for intraportal injection (Table 3). However, Soderlund et al. showed that necrosis of the graft was evident on the first day, and was progressive to day three; the necrotic areas contained polymorphonuclear cells (95). Spreading the islets over a larger surface area reduces the risk of ischemic injury (91, 95).

The immense advantage of porcine islets over human islets is their abundant availability; this advantage does not appear to have been fully utilized experimentally.

Non-immunological responses to transplanted islets

Data provided largely by the Uppsala group indicate that islets injected intraportally, whether they be allo (102) or xeno (94), are subject to IBMIR, which is manifest by activation and consumption of platelets, activation of neutrophils and monocytes, and activation of the coagulation and complement systems (94, 103). The prior administration of soluble complement receptor-1 reduced rapid islet destruction both in vivo and in vitro (94), supporting the hypothesis that complement activation plays a major role in IBMIR. However, others have reported long-term survival of porcine islets in nonhuman primates without the need for specific treatment for IBMIR (89). The immediate and significant loss of islets from IBMIR may possibly be reduced by some of the agents primarily administered as immunosuppressants, such as those that inhibit cytokine release.

Immunological response to transplanted islets

Both humoral and cellular rejection of the transplanted islets has been described (Table 3).

Humoral response

Anti-Gal is the major preformed antibody directed against pig epitopes in humans. However, a lack of Gal expression in the adult pancreas, apart from the lining of blood vessels and biliary ducts, has been reported by several groups (15, 16, 18, 19), with occasional Gal expression by others (17, 21, 104). In contrast, Gal expression on neonatal (20, 21) and fetal (17) islets is high. Fetal islets from wild-type pigs are therefore presumed to be more susceptible to humoral injury than adult islets (17).

No study of islet xenotransplantation in primates to date has used islets from pigs homozygous for α1,3galactosyltransferase gene-knockout. The availability of these pigs (105-107) has made it important to investigate the potential role in graft destruction played by antibodies directed to other (nonGal) pig antigens (104, 108, 109). Hanganutziu-Deicher (HD) antigens and other sialic acid antigens have been shown to account for significant antigenicity of adult pig islets in vitro (104). Furthermore, nonGal and nonHD antigens have been shown to be of importance. Komodo et al. transplanted islets from N-acetylglucosaminyltransferase-III (GnT III) pigs, in which both Gal and nonGal antigen expression is down-regulated (110, 111), under the renal capsule of monkeys. These modified islets survived longer than wild-type islets. Since monkeys, unlike humans, express HD antigens (108), this observation suggests the existence of antibodies to nonGal, nonHD antigens (109).

Organs from pigs transgenic for the human complement regulatory protein, decay-accelerating factor (hDAF) are less susceptible to hyperacute rejection after transplantation into primates, as the porcine endothelium is protected to some extent from complement-mediated injury after contact with human blood. However, both fetal and adult islets from hDAF transgenic pigs generated strong cytotoxic and inflammatory responses that were equal to those generated by islets from wild-type pigs, suggesting that hDAF was not expressed strongly

Table 3: Experience with pig-to-nonhuman primate islet transplantation

First author	Reference	Donor islets	Recipient (n, when stated) (diabetic status)	Site and number of islets transplanted (when stated) (IE or ICC)
Kirchhof	(82)	WT adult	Rhesus (8) (6 STZ-diabetic, 2 non-diabetic)	Intraportal, 20 000/kg
Jie	(89)	WT adult	Cyno (3) (STZ-diabetic)	Intraportal, 25 000/kg
			Cyno (4) (STZ-diabetic)	Intraportal, 25 000/kg
			Cyno (4) (STZ-diabetic)	Intraportal, 25 000/kg
			Cyno (1) (STZ-diabetic)	Intraportal, 25 000/kg
Rijkelijkhuizen	(91)	WT adult	Cyno (4) (non-diabetic)	Kidney capsule, 1 500-9 500 on 4 poles
			Rhesus (4) (non-diabetic)	Kidney capsule; 1 500-9 500 on 4 poles (n=4), Intraportal; 2 000-8 500 (n=3)
Buhler	(92)	WT adult	Baboon (3) (pancreatectomized)	Intraportal; 10 000/kg
		Miniature swine adult	Baboon (2) (non-diabetic)	Intraportal; 14 000/kg and 32 000/kg
Cantarovich	(93)	WT adult	Baboon (4) (non-diabetic) Cyno (1) (non-diabetic)	Intraportal, 60-180 000 (n=5), Additional subcutaneous islets, 2000 (n=3)
Bennet	(94)	WT adult	Cyno (4) (non-diabetic)	Intraportal 19 700-20 000/kg
			Cyno (4) (non-diabetic)	
Soderlund	(95)	WT fetal	Cyno (8) (non-diabetic)	Kidney capsule, 4 000
			Cyno (6) (non-diabetic)	Kidney capsule, 4 000
Mandel	(96)	hDAF fetal	Cyno (non-diabetic)	Kidney capsule
		WT fetal	Cyno (non-diabetic)	Kidney capsule
Sun	(66)	WT adult	Cyno (11) (spontaneously diabetic)	Intraperitoneally; Unencapsulated (n=2) and Encapsulated (n=9); 1-3 times 30-70 000

Abbreviations: ATG= antithymocyte globulin; C-pep= porcine C-peptide; CyA= cyclosporine; Cyc= cyclophosphamide; cyno= cynomolgus monkey; CVF= cobra venom factor; d= posttransplant day; EVL= everolimus; h= hour; ICC= islet cell clusters; IE= islet equivalents, LEF= leflunomide; LF 195= deoxyspergualin analog; min= minute; MMF= mycophenolate mofetil; MMN= monomorphonuclear cells; PMN= polymorphonuclear cells; rhesus= rhesus monkey; STZ= streptozotocin; WT= wild type

Immunosuppressive regimen	Result
None	Serum C-pep lost in 12-72h Histology: 12-24 h: complement and Ab deposition; 48-72 h: CD3/4/8 T cell deposition.
Anti-CD25, FTY720, EVL	Serum C-pep for 39, 47 and 53 d
Anti-CD25, FTY720, EVL, anti-CD154	Serum C-pep for 57, 68, >73 and >187 d
Anti-CD25, FTY720, EVL, anti-CD154, LEF	Serum C-pep for >76, >111,>140 and >145 d
Anti-CD25, FK 506 , EVL, anti-CD154, LEF	Serum C-pep for > 158 d
Cyc, CyA, prednisolone	Histology: up till day 11 little infiltration (5%). Serum C-pep <0.33 ng/mL
ATG, anti-IL 2 receptor Ab, CyA, prednisolon	Histology kidney: up till day 53 without infiltration. Serum C-pep <0.33 ng/mL No islets in liver detected
ATG, Cyc, azathioprine	Normoglycemia for 15-2 h
Splenectomy, Whole body+ thymic irradiation, ATG, anti Gal immunoadsorption, CVF, CyA, MMF, prednisolone, anti-CD154, donor peripheral blood progenitor cell infusion	Serum C-pep till d 3 and 5
ATG, CyA, LF 195, MMF, prednisolone	Serum C-pep for 2 d (n=1) Serum C-pep for 1 d (n=1) Serum C-pep for 2 h (n=2) No serum C-pep (n=1)
sCR1	Reduced insulin release, suggesting less islet cell damage
None	Plasma insulin increased 60 fold first 10 min
None	Histology: d 1/3/6: increase in PMN cells (mainly macrophages), T cells (CD8>CD4) and necrosis (few B cells and NK cells).
CyA, 15-deoxyspergualin	Histology: d 6: fewer T cells and necrosis compared to untreated monkeys. d 12: CD 8 cells
Non-immunosuppressed and immunosuppressed with CyA, steroids, Cyc / Brequinar	Non-immunosuppressed: Histology: d 3; minimal infiltration, d 7; massive infiltration PMN and MMN cells. Immunosuppressed: Histology: d 7; grafts present (not all recipients). Histological survival > 40 d.
None	Encapsulated islets: Insulin independence for 120-804 d and serum C-pep (n=7)

on the islets (112). It will be necessary to develop promoters that are islet-specific if islets, as opposed to vascular endothelial cells, are to be protected by this transgenic approach.

Cellular response

Both in vitro and in vivo studies show cellular rejection of transplanted islets to be important (82, 91, 113) (Table 3). Recently, Kirchhof et al. showed that adult islets transplanted intraportally into diabetic non-immunosuppressed monkeys were subject to acute cellular rejection. Although early IgM binding and complement deposition was reported, islets were not hyperacutely rejected. Early reversal of hyperglycemia was demonstrated, together with porcine C-peptide production. The islets were subject to $CD4^+$ and $CD8^+$ cell infiltration, that progressively increased until euthanasia after 72 hrs (57).

Also in non-immunosuppressed monkeys, the Uppsala group showed that subcapsular islets were infiltrated by mononuclear cells, mostly T cells and macrophages, and no immunoglobulin or complement deposition was detected (114, 115).

Soderlund et al. demonstrated also no immunoglobulin deposition in subcapsular fetal islet grafts, but macrophages and T cells ($CD8^+$>$CD4^+$) infiltrated the entire graft, though this followed initial necrosis, possibly a result of ischemia (95). Histology from subrenal capsular and intraportal grafts showed increasing T cell infiltration before graft loss, with few B cells, macrophages and NK cells (91).

Lalain et al. co-incubated pig islets with baboon and human peripheral blood mononuclear cells (PBMC), and found early a greater $CD4^+$ than $CD8^+$ T cell response, suggesting more Th2 than Th1 activity (113). The importance of $CD4^+$ T cells was also demonstrated by Friedman and colleagues who studied the human lymphocyte response to fetal porcine islets in a recombinase-activating gene-deficient (RAG) mouse model (116). Cantarovich et al. suggested that T cell-dependent humoral rejection occurs; after an initial infiltration with macrophages, they found T cell infiltrates in the graft, and strong antibody responses (93). Islets transplanted into the sternomastoid muscle were also reported to be destroyed by macrophages and mononuclear cells (92).

The evidence to date is inconclusive, but rejection of pig islets in nonhuman primates is probably a result of an innate response, manifest by macrophage infiltration and possibly antibody- and complement-mediated injury, followed by an adaptive response, manifest by a major T cell response.

Suppression of the immune response

Various immunosuppressive regimens have been tested in pig-to-primate islet experiments with varying results (Table 3). In all studies in which a multi-drug therapy approach has been incorporated, it is difficult to determine the effect of any one agent.

Suppression of the humoral response

The administration of cobra venom factor resulted in complete depletion of complement (CH50 levels 0%) although its effect on islet survival was not clear (92). Soluble complement receptor-1 reduced IBMIR significantly (94). IBMIR has also been partially prevented by the administration of dextran sulfate in both in vitro and in vivo models (117, 118).
Extracorporeal immunoadsorption of anti-Gal antibody, which is a time-consuming procedure, has been used to prevent antibody-mediated hyperacute rejection (92), but its effect is transient, lasting for only a few days (119). In view of the relative or complete absence of Gal expression on adult pig islets, it is unlikely that this immunoadsorption played any role in extending islet graft survival.

Suppression of the cellular response

Cyclosporine has been widely used in the past, but, because of nephrotoxic side effects, newer drugs are increasingly being used. Antithymocyte globulin and/or an anti-IL2 receptor antagonist are widely used (Table 3). When these drugs were added to a cyclosporine-based regimen, survival of porcine islets in monkeys was extended significantly; cellular infiltration in subrenal capsular islet grafts was delayed from 11 to 53 days (91).

Immunosuppressive regimens containing sirolimus and tacrolimus, as used in the Edmonton protocol (1), have been of interest. Unfortunately, tacrolimus is diabetogenic, even when low levels of the drug (4-6 ng/ml) are maintained in the blood. Furthermore, it has been shown in rats that the Edmonton regimen induces marked insulin resistance and beta-cell toxicity (120). Alternatives to tacrolimus should be explored (121). Mycophenolate mofetil has not been used frequently in islet transplantation experiments in nonhuman primates, but is a potential alternative to tacrolimus as it is a T and B cell suppressor and less diabetogenic (121, 122). Corticosteroids used to be constituents of immunosuppressive regimens (91-93, 96), but, because of side effects, in particular diabetogenicity, have now been omitted.

The most successful results to date have been obtained using a regimen that includes several newer agents - FTY 720, an anti-CD154 mAb, and leflunomide – in addition to some more conventional agents (89). When an anti-CD154 mAb was added to the immunosuppressive regimen, survival of the islets increased from 53 days to >187 days (89) (Table 3). These results correlate with the long-term islet survival achieved in an anti-CD 154 mAb-based regimen in allotransplantation experiments (123, 124).

Assessment of survival of islets after transplantation

Porcine C-peptide levels (9, 66, 82, 89, 91-93, 98) and histology of the graft (Table 3) are the most important indicators of graft viability. The intravenous glucose tolerance test, and the requirement for exogenous insulin (98) have also been used. Arginine and glucagon stimulation tests may also be valuable.

COMMENT

At the present time, it would seem that adult porcine islets have the best potential, from both logistic and immunologic perspectives, to reverse diabetes in primates. Islet isolation techniques have improved sufficiently so that large numbers of good-quality adult porcine islets can be obtained. Refinements in the induction and maintenance of STZ-induced diabetes in nonhuman primates have provided a useful experimental model, although it remains uncertain whether STZ induces irreversible diabetes.

However, progress in extending survival of transplanted pig islets in nonhuman primates has been slow, although encouraging results have recently been reported (89). With the relative plethora of established and novel immunosuppressive agents now available, one major problem will be to determine the optimal combination. Particularly in the treatment of a disease such as diabetes, that is not rapidly fatal, there is a need to establish clinically-applicable immunosuppressive regimens that are not associated with life-threatening side effects. There will almost certainly be a move away from calcineurin inhibitors, that are diabetogenic, towards newer agents, such as FTY 720, leflunomide, and biological agents that inhibit costimulation.

As we have outlined in this review, experimental models have been established in which these novel agents can be investigated. However, questions remain, and details of these models need to be adequately addressed.

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Chapter 3
Antibodies directed to pig nonGal antigens in naïve and sensitized baboons

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ABSTRACT

Background: As pigs homozygous for α1,3-galactosyltransferase gene-knockout (GT-KO) are available, primate antibodies to pig nonGal antigens can be studied.
Methods: Sera from 56 baboons were tested for binding of IgM and IgG to peripheral blood mononuclear cells (PBMC) from both wild-type (WT) and GT-KO pigs by flow cytometry. Complement-dependent cytotoxicity was measured in 39 sera. Antibody and cytotoxicity responses were measured in two baboons exposed to a GT-KO pig heart, one not immunosuppressed, and one that received only cobra venom factor.
Results: IgM and IgG bound to 95% and 79% of WT PBMC, and 32% and 9% GT-KO PBMC, respectively (WT versus GT-KO, $p<0.01$). Whereas 97% of sera were cytotoxic to WT PBMC, only 64% were cytotoxic to GT-KO PBMC, and the level of cytotoxicity was less (mean 60% versus 25% lysis, $p<0.05$). In the two baboons exposed to GT-KO hearts, anti-nonGal antibodies increased markedly, peaking after 2 (IgM) and 3 (IgG) weeks, associated with an increase in lysis of GT-KO PBMC.
Conclusions: Almost 50% of baboons have preformed cytotoxic antibodies to GT-KO PBMC. After GT-KO organ transplantation, if an elicited antibody response develops, it is likely to cause rapid graft rejection.

INTRODUCTION

The recent availability of pigs homozygous for α1,3-galactosyltransferase gene-knockout (GT-KO) (1, 2) has enabled pig-to-baboon organ transplantation to be carried out in the absence of expression of the pig Galα1,3Gal (Gal) epitopes that are known to be important targets for primate anti-pig antibodies (3-6). Transplantation of hearts (7) and kidneys (8) from GT-KO miniature swine into immunosuppressed baboons that expressed either no or very low levels of preformed antibodies pre-transplantation against antigens on GT-KO pig peripheral blood mononuclear cells (PBMC) (nonGal antigens) was followed by relatively prolonged graft survival, extending to 179 days (heart) and 83 days (kidney), with graft failure largely a result of the development of a thrombotic microangiopathy that may be a form of delayed antibody-mediated rejection. At graft excision, patchy IgM, IgG, and C4d deposition was seen in the majority of the grafts.

Although Zhu has reported a varying incidence of anti-nonGal antibodies (against α-galactosidase-treated wild-type pig red blood cells) in 10 healthy humans (9), there have hitherto been no other definitive reports on the incidence of anti-nonGal antibodies in any species of primate or of the extent of cytotoxicity associated with these antibodies towards pig cells. We here report on the incidence and cytotoxicity of preformed antibodies directed to nonGal antigens on GT-KO pig PBMC in immunologically-naïve baboons. We also report

on the development of elicited antibodies to nonGal antigens after baboon exposure to hearts from GT-KO pigs.

METHODS

Animals

Serum samples were prepared from blood collected from 56 immunologically-naïve baboons (not intentionally exposed to pig cells or tissues) (*Papio anubis* or *Papio hamadryas*) of all blood groups (A, B, AB); the sera came from baboons from various suppliers in the US. To detect the presence of anti-Gal and anti-nonGal antibodies in these sera by flow cytometry, PBMC as targets were collected from wild-type (WT) and GT-KO pigs (Revivicor, Inc., Blacksburg, VA) (1), both of Large White/Landrace/Duroc cross-breed, and all of blood type O. Although the WT and GT-KO pigs were from the same genetic background, they were not from identical clones; however, the only major difference was in the absence of Gal antigens in the GT-KO pigs. Sufficient sera were available from 39 baboons to perform cytotoxicity assays, using the same pig cells.

All animal care procedures were in accordance with the *Principles of Laboratory Animal Care* formulated by the National Society for Medical Research and the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication No. 86-23, revised 1985). Protocols were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh.

Isolation of Porcine PBMC

PBMC from several different pigs (WT and GT-KO) were isolated from heparinized blood by centrifugation on Ficoll-Paque PLUS (Amersham Biosciences, Piscataway, NJ). The PBMC were not pooled, but PBMC from a single pig were used for multiple sera. The total mononuclear cell fraction was then washed twice with RPMI 1640 media (Gibco, Grand Island, NY), filtered and resuspended in FACS Buffer (PBS containing 1% bovine serum albumin [Gibco] and 0.1% NaN_3) for flow cytometry or in RPMI medium (including 10% FBS) for cytotoxicity assay.

Flow Cytometry

Baboon serum samples (n = 56) were incubated for 30min at 56˚C to inactivate complement. Isolated PBMC ($1x10^6$ cells/each tube) were incubated with 20µL of baboon serum from each sample (20% serum final concentration) or 20µL of FACS buffer as a control for 30min at 4˚C. After incubation, the cells were washed twice in 2mL FACS buffer and centrifuged at 1800rpm for 5min. The supernatant was discarded. To prevent non-specific binding, 10µL of goat serum (10% concentration) was added to all the tubes. Detection of IgG or IgM binding

was performed by further incubating the serum with FITC-conjugated goat anti-human IgG (γ chain-specific) at 1:50 dilution or IgM (μ chain-specific) at 1:200 dilution (Zymed Laboratories, San Francisco, CA) for 30min in the dark at 4˚C. The samples were washed twice, and the cells resuspended with FACS buffer. Data acquisition was performed with FACScan (Becton Dickinson; Mountain View, CA). Dead cells were excluded from the analysis by forward-scatter and propidium iodide staining. IgG and IgM anti-pig antibodies were determined by indirect immunoflorescence intensity. Measurement of mean florescence intensity (MFI) was performed on viable cells. Measurement of indirect immunoflorescence intensity was accomplished by Cell Quest software and converted into MFI by WinMDI software.

Complement-Dependent Cytotoxicity Assay

Baboon serum samples (*n* = 39) were incubated for 30min at 56˚C to inactivate complement. The samples were four-fold serially diluted with RPMI 1640 media (+10% FBS; Gibco) to final concentrations of 50%, 12.5%, 3.125%, and 0.78%. As an interplate control, serum from a single human was diluted in the same manner and used on all plates. Cells were loaded into flat-bottomed 96-well plates at a density of $1x10^6$ cells/well (suspended in RPMI 1640 media + 10% FBS). The cells were centrifuged at 1800rpm for 7min, the supernatant discarded, and the cells incubated with 100μL of diluted baboon or human (interplate control) serum for 30min at 4°C.

After incubation, the cells were washed and further incubated with 100μL of 10% rabbit HLA-ABC serum (Sigma), as a source of complement, for 45min at 37°C. Negative control wells (100% live cells) were achieved by adding only complement to wells containing either WT or GT-KO cells. Positive control wells (0% live cells) were achieved by adding a detergent (polyoxyethylene sorbitan monolaurate; Sigma). Additional negative controls were employed; these comprised either cells alone or cells + serum in wells in which no complement or detergent was added.

Following incubation, the cells were washed and resuspended in 100μL of RPMI 1640 (+10% FBS) and 20μL of tetrazolium compound (CellTiter 96® Aqueous One Solution Reagent; Promega, Madison, WI) and incubated for 15h, for the development of color, at 37˚C in a 5% CO_2 humidified atmosphere. Cell viability was assessed by the capacity of live cells to reduce tetrazolium salts into formazan, and the color produced was measured (absorbance) using a plate reader (Bio-Tek Instruments, Winooski, VT) at a wavelength of 450nm. The assumption made in this assay was that a cell that is incapable of producing color is a dead cell.

Cell viability was calculated using the following formula:

$$\% \text{ live cells} = [(A-B)/(C-B)] \times 100,$$

where A equals the absorbance of cells (+ serum and complement), B equals the absorbance of cells (+ detergent), and C equals the absorbance of cells (+ complement only).

Results were expressed as the percentage of dead cells (% cytotoxicity = 100 - % viability). Complement-dependent cytotoxicity values of the varying serum concentrations (50%, 12.5%, 3.125%, and 0.78%) were calculated, and a curve was generated for each baboon sample. Results were considered acceptable based on graded concentration of human interplate control serum that was added to every plate. Lysis of PBMC of <10% was considered of doubtful relevance.

Data Analysis

Statistical analysis of data was performed using Mann-Whitney tests. A *p* value of <0.05 was considered to be statistically significant. Correlation of cytotoxicity and MFI was assessed by linear regression

Heterotopic Heart Transplantation

One non-immunosuppressed baboon and one baboon that received daily therapy with only cobra venom factor to deplete complement (both of blood type B) underwent heterotopic transplantation (in the abdomen) of hearts from GT-KO pigs (blood type O). No other therapy, except modest anticoagulation with heparin (to maintain the partial thromboplastin time at >100sec), was administered. In the non-immunosuppressed baboon, the heart was electively excised after 150min (as it proved too large to allow satisfactory closure of the baboon's abdomen), and the graft in the second baboon was excised after being rejected on day 8. In both cases, remnants of the pig aorta and pulmonary artery were left *in situ.* The antibody responses of the two baboons were followed by flow cytometry and cytotoxicity assay for >2 months and >1 month, respectively. In these assays, third party (not donor-specific) PBMC were used.

RESULTS

Reactivity of Serum from Naïve Baboons with PBMC from WT and GT-KO Pigs

Binding of baboon IgM and IgG to pig PBMC

The presence of preformed xenoreactive antibodies in sera from naïve baboons was assessed by flow cytometry. All but 3 of the 56 sera (95%) showed detectable IgM binding to WT PBMC, and 44 sera (79%) showed IgG binding, with binding (IgM and/or IgG) seen in 55 sera (98%) (Figure 1A). In contrast, only 18 of the 56 sera (32%) showed detectable IgM binding to GT-KO PBMC, and only 5 showed IgG binding (9%); binding was detected in 20 sera (36%) (Figure 1A). The incidence of binding of baboon IgM and IgG antibodies to GT-KO PBMC was therefore only 34% and 11%, respectively (overall 36%), of that to WT PBMC.

The mean MFI of IgM binding to PBMC was 6.4 (WT) and 4.0 (GT-KO), respectively ($p<0.01$) (Figure 1B). The mean MFI of IgG binding was 6.2 (WT) and 3.3 (GT-KO), respectively ($p<0.01$). No baboon showed a high MFI of IgM binding to GT-KO PBMC, although five did show a moderately high MFI of IgG binding (Figure 1A).

These results suggest that the greater binding of baboon sera to WT PBMC was due to Gal-specific antibodies, and the residual binding to GT-KO PBMC was due to the presence of antibodies to nonGal antigens.

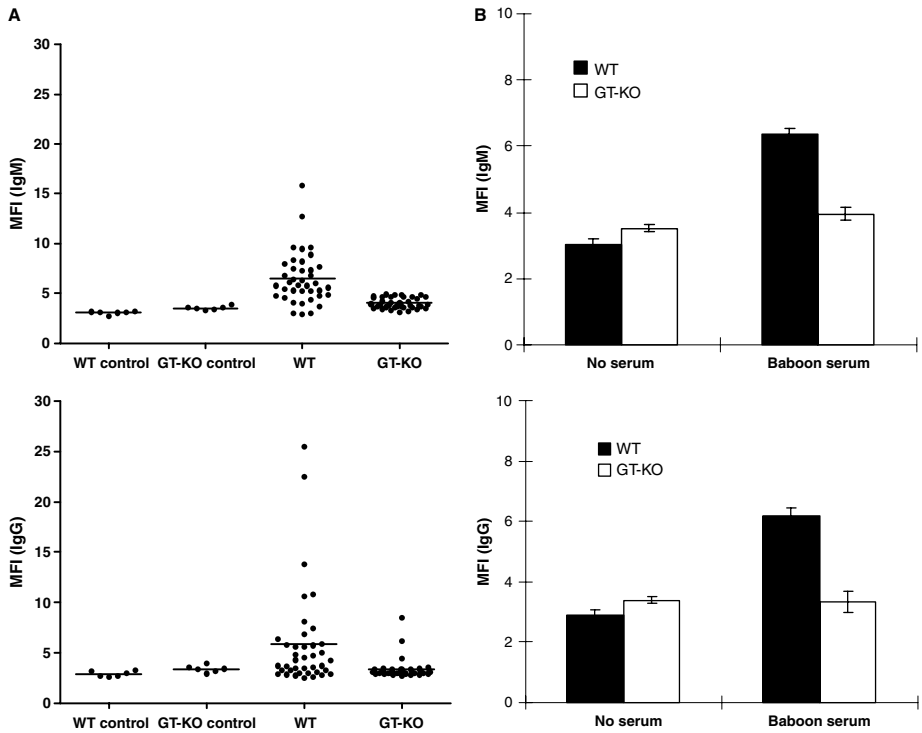

Figure 1:
Binding of baboon preformed xenoreactive antibodies to WT and GT-KO pig PBMC [MFI = mean fluorescence intensity]. **(A)** Distribution of serum reactivity of 56 baboons against WT or GT-KO PBMC, IgM (top) and IgG (bottom). Mean reactivity of each group is indicated by a line. Control is FITC-conjugated secondary antibody only. **(B)** Mean reactivity (MFI levels ± S.D.) of serum samples (n = 56) against WT or GT-KO PBMC, IgM (top) and IgG (bottom). Statistically higher ($p<0.01$) reactivity of both IgM and IgG against WT PBMC versus GT-KO PBMC was observed.

Complement-dependent cytotoxicity of baboon sera to pig PBMC

Of the 56 baboon serum samples screened for antibody binding by flow cytometry, only 39 were available in sufficient quantity to allow complement-mediated cytotoxicity assay. At 50% dilution, 38 of the 39 sera (97%) were cytotoxic to WT PBMC, whereas only 25 (64%) were cytotoxic to GT-KO PBMC (Figure 2A). Furthermore, the sera caused greater lysis of PBMC from WT than from GT-KO pigs ($p<0.01$) (Figures 2A and B); at 50% dilution, the mean lysis of PBMC was reduced from approximately 60% (WT) to 25% (GT-KO). Sera from the five baboons that had high MFI of IgG binding to GT-KO PBMC did not show particularly high cytotoxicity levels.

We concluded that approximately two-thirds of the naïve baboon sera tested contained anti-nonGal antibodies that could mediate lysis of PBMC from GT-KO pigs, though the level of cytotoxicity was generally weaker than that of WT PBMC.

Correlation between binding of IgM or IgG and serum cytotoxicity

Comparisons were made between the flow cytometric data and cytotoxicity of the 39 sera to WT and GT-KO PBMC. IgM and IgG binding data were correlated with serum cytotoxicity. Although a trend towards an increase in cytotoxicity to WT PBMC was observed to accompany both higher IgM and IgG MFI, this trend did not reach statistical significance (data not shown). No correlation was found between MFI (IgM or IgG) and cytotoxicity to GT-KO PBMC.

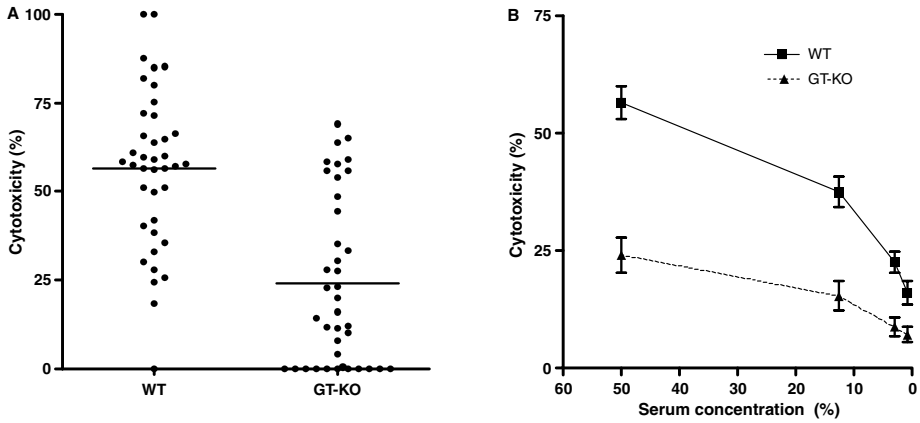

Figure 2:

(A) Ability of individual baboon serum samples (at 50% dilution, n = 39) to cause lysis of PBMC from WT or GT-KO pigs; the mean cytotoxicity is indicated by lines. Significantly higher lysis of WT PBMC was observed ($p<0.01$). **(B)** Mean cytotoxicity of baboon sera at various dilutions against PBMC from either WT or GT-KO pigs.

Reactivity of Sera from Baboons after Exposure to Transplanted GT-KO Pig Hearts

To investigate the extent of the elicited antibody response to nonGal antigens, serum samples were collected from two baboons before and at different time periods after heterotopic transplantation of hearts from GT-KO pigs. In one non-immunosuppressed baboon, the heart remained *in situ* for only 150min, before being electively excised while beating (as it was too large to close the abdomen satisfactorily). In the other baboon, that received only cobra venom factor, the heart was excised after rejection on day 8.

In the first baboon, there was little difference in baseline anti-pig IgM or IgG reactivity to WT or (third party) GT-KO pig PBMC (Figure 3). A classical immune response was mounted against the pig organ, with IgM levels peaking after approximately 2 weeks (Figure 3A), while IgG peaked after approximately 3 weeks (Figure 3B). There was a slow fall in the level of anti-pig antibody binding to GT-KO PBMC over the next several weeks. The response was against nonGal antigens, since, using an ELISA with which we have considerable experience (10), the anti-Gal antibody level did not change (Figure 4), confirming the absence of Gal expression on the GT-KO pig hearts (1).

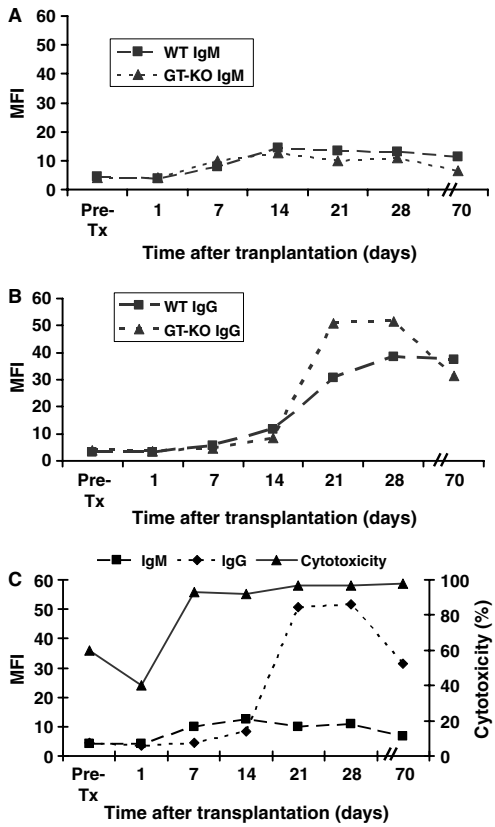

Figure 3:
Flow cytometry data showing **(A)** IgM and **(B)** IgG responses (to both WT and GT-KO pig PBMC) in a baboon that was exposed to a GT-KO pig heart for 150min (on Day 0). One hour following heart transplantation, the levels of IgM and IgG binding were similar to pre-transplant levels. An increase in IgM binding was observed 4 days after exposure to the heart, and peaked between days 9 and 14, whereas the IgG response was not detected until day 7, peaking between days 16 and 21; the increase in IgG was rather faster and greater to GT-KO than to WT PBMC. **(C)** A cytotoxicity assay curve against (third party) GT-KO PBMC was generated. Lysis of GT-KO PBMC correlated with the increase in IgM, but was observed approximately one week before any increase in IgG. One week after transplantation, 100% lysis of GT-KO PBMC was detected, which remained high for more than 2 months.

At 50% serum concentration, pre-transplant lysis of GT-KO PBMC (60%) (Figure 3C) was less than that of WT PBMC (80%) (not shown). One week after transient exposure to the GT-KO pig heart, lysis to both WT and GT-KO PBMC had increased to 100%, and remained high for up to 10 weeks. The initial increase in cell lysis of GT-KO PBMC appeared to correlate with the rise in IgM, but preceded the rise in IgG.

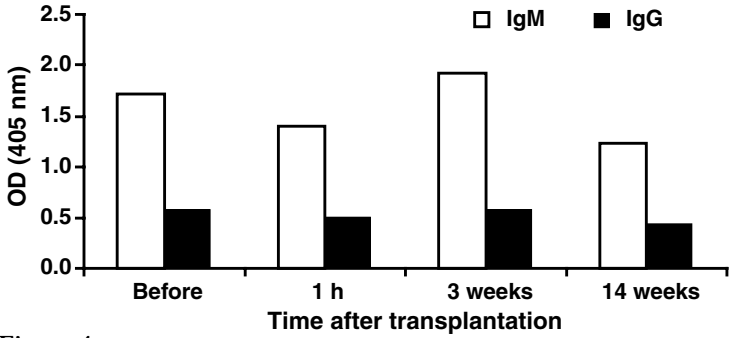

Figure 4:
Anti-Gal antibody (IgM and IgG) levels by ELISA for the first baboon at a serum concentration of 1:50. The GT-KO pig heart was excised after 150 minutes, and the baboon was followed 14 weeks. No antibody response to Gal was documented.

In the second baboon, the timing of the increases in anti-nonGal IgM and IgG and in cell lysis was comparable to that seen in the first baboon, with IgM peaking after the first week, IgG peaking at 2-3 weeks, and increased cytotoxicity being documented within 7 days of transplantation (not shown). However, the magnitude of the increases in the levels of both IgM and IgG was greater in this baboon, presumably because exposure to the GT-KO pig heart had been more prolonged (8 days versus 150min) and because the heart underwent acute humoral xenograft rejection before being excised. There was a small increase in anti-Gal IgG level on day 8 (day of rejection) with the MFI increasing from 1.0 to 2.0, followed by a reduction to 1.7 by day 35; this did not indicate sensitization to Gal (although sensitization to Gal molecules on CVF has been reported in non-immunosuppressed baboons [11]).

In both baboons, after exposure to a GT-KO pig heart, *in vitro* increased binding of elicited IgG to (third party) GT-KO PBMC occurred a few days earlier than to WT PBMC (Figure 3B), possibly as a response to a greater intensity of nonGal antigens on the GT-KO PBMC than on the WT PBMC (where the presence of Gal epitopes may have reduced the number of nonGal antigens exposed, i.e., the density of nonGal antigens may be lower on WT PBMC). The difference in MFI between GT-KO and WT PBMC (Figure 3B) was not associated with a response to donor-specific transplantation antigens, since third party PBMC were used for both the GT-KO and WT studies.

DISCUSSION

Organ transplantation from a WT pig into a nonhuman primate usually results in hyperacute rejection within minutes to hours (12). The primary role of anti-Gal antibodies in this response, first reported in 1992 (4-6), has recently been overcome by the breeding of pigs homozygous for GT-KO (1, 2). However, although the transplantation of an organ from a GT-KO pig into an immunosuppressed nonhuman primate allows extended graft survival (7), the graft eventually fails from the development of a thrombotic microangiopathy that may be a form of delayed antibody-mediated rejection. The focal deposition of IgM, IgG, and C4d on the graft suggests that a component of this process may involve the binding of anti-nonGal antibodies to the pig vascular endothelial cells. These antibodies could be either preformed (in naïve baboons) or elicited (in baboons sensitized by exposure to GT-KO pig antigens).

The present study investigated the incidence of preformed anti-nonGal antibodies in the serum of naïve baboons, and assessed whether these antibodies are cytotoxic to pig PBMC lacking the Gal epitope. The results were compared with reactivity of anti-pig antibodies to WT pig PBMC.

Whereas all but one of 56 baboons had preformed antibodies, mainly IgM, to WT pig PBMC, less than half had such antibodies to GT-KO PBMC. Moreover, the cytotoxicity associated with anti-nonGal antibodies alone (to GT-KO PBMC) was significantly weaker than that associated with anti-Gal + anti-nonGal antibodies (to WT PBMC). We were, however, unable to show a statistical correlation between the presence of these antibodies and serum cytotoxicity. The complement-dependent cytotoxicity assay appeared to be a more sensitive indicator of the presence of anti-pig antibody than flow cytometry. Our data, therefore, suggest that GT-KO pigs are preferable to WT pigs as sources of organs for transplantation into primates. Nevertheless, anti-nonGal antibodies initiated a cytotoxic response; *in vivo,* this would be expected to jeopardize long-term viability of a pig organ graft. No baboon showed a high MFI of IgM binding to GT-KO PBMC, although five did show a moderately high MFI of IgG binding (Figure 1A). Although these ‘outliers’ did not demonstrate increased serum cytotoxicity to GT-KO PBMC, this needs to be investigated further, since this observation may be of ‘clinical’ importance.

One possible limitation of our study is that only PBMC were used as target cells, whereas vascular endothelial cells might have provided different, or more biologically relevant, nonGal epitopes, although there is no definite evidence for this (13). We are currently investigating this in our laboratory; preliminary results indicate that binding to PBMC may actually be *greater* than to vascular endothelial cells (Hara H, et al, unpublished), suggesting that PBMC provide a biologically relevant target.

We have also investigated the presence of preformed anti-nonGal antibodies in humans (14); these data will be reported separately. However, baboons would appear to have a slightly lower level of anti-nonGal antibodies than humans, both on flow cytometry and complement-dependent cytotoxicity assay. This difference will need to be considered when assessing the results of GT-KO pig organ transplantation in the pig-to-baboon model.

The capacity of elicited antibodies, collected from the sera of two baboons exposed to GT-KO pig hearts, to bind to GT-KO PBMC, and to lyse those cells, was also assessed. Immunoglobulins, predominantly of the IgG isotype and specific for undetermined nonGal antigens, reached high levels post-transplantation, and were associated with a significant increase in cell lysis. In the case of the baboon that received cobra venom factor therapy, the elicited antibody response, restricted to nonGal antigens, was associated with rejection of the graft. The timing of the elicited antibody response (beginning by day 7) was similar to that seen previously when baboons were exposed to WT pig antigens, but the amplitude of the response would appear to be reduced (15,16).

We found no evidence of continued expression of Gal in the GT-KO pigs, e.g., as a result of iGb3 synthetase activity, as has been suggested by others (17,18) since, after exposure to GT-KO pig hearts, and despite sensitization to nonGal antigens, there was no increase in the level of anti-Gal antibodies. Previous *in vitro* studies involving WT cells and either immunoadsorption of primate sera or treatment of the cells with a galactosidase have suggested that antibodies to nonGal antigens represent only 10-30% of anti-pig IgM and 20-50% of IgG antibodies in naïve sera (reviewed in 19). The data observed using GT-KO PBMC in the present study would largely agree with these estimates, but would suggest that anti-nonGal antibodies may represent a greater proportion of anti-pig antibodies in some baboons.

Acute humoral xenograft rejection associated with the presence of preformed or elicited anti-nonGal antibodies might therefore be addressed by further modification of the pig to reduce the expression of nonGal antigens. The identification of nonGal specificities would therefore be an important step forward. However, the nature of the nonGal antigens expressed on GT-KO pig vascular endothelium or PBMC is currently uncertain, although several carbohydrates are candidates (20, 21). It has been demonstrated that there is some rearrangement of the carbohydrate pattern of the vascular endothelium in mice after GT-KO (22), and neoantigens might be exposed, to which some preformed antibodies may be directed. N-glycolylneuraminic acid epitopes, so-called Hanganutziu-Deicher antigens, are widely expressed on the endothelial cells of all mammals except humans, and are considered to be potential porcine targets for preformed and elicited anti-nonGal antibodies in humans (23-25), but not in baboons, which express these epitopes. It is equally likely, however, that the targets for elicited anti-nonGal Abs are proteins, rather than carbohydrates.

If there are multiple nonGal epitopes against which primates have preformed antibodies, then gene-knockout may not prove to be a feasible solution. However, the lower level of endothelial cell activation and cytotoxicity associated with anti-nonGal antibodies, compared with anti-Gal antibodies, may allow some protection to be achieved by the presence of one or more human complement-regulatory protein. Although transgenesis for human decay-accelerating factor or other complement-regulatory proteins has had only a relatively limited protective effect against anti-Gal antibodies, this approach may be more successful against the weaker anti-nonGal antibodies.

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Chapter 4
Allosensitized humans are at no greater risk of humoral rejection of GT-KO pig organs than other humans

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ABSTRACT

Background: The availability of pigs homozygous for α1,3-galactosyltransferase gene-knockout (GT-KO) has enabled study of the incidence and cytotoxicity of primate antibodies directed to antigens other than Galα1,3Gal (Gal), termed nonGal antigens.
Methods: Sera from 27 healthy humans and 31 patients awaiting renal allotransplantation, who were either unsensitized (PRA<10%) or allosensitized (PRA>70%), were tested by flow cytometry for binding of IgM and IgG to peripheral blood mononuclear cells (PBMC) from both wild-type (WT) and GT-KO pigs. Complement-dependent cytotoxicity to WT and GT-KO PBMC was also measured.
Results: IgM and IgG from all 27 (100%) healthy human sera bound to WT PBMC, while 78% and 63% of these sera had IgM and IgG that bound to GT-KO PBMC, respectively. Mean binding to WT PBMC was significantly greater than to GT-KO PBMC. Whereas 100% of sera were cytotoxic to WT PBMC, only 61% were cytotoxic to GT-KO PBMC, and the extent of lysis was significantly less. Neither mean binding of IgM and IgG nor cytotoxicity of unsensitized and allosensitized sera to WT and GT-KO PBMC was significantly different to that of healthy sera.
Conclusions: More than half of the healthy humans tested had cytotoxic antibodies to GT-KO PBMC, but allosensitized patients will be at no greater risk of rejecting a pig xenograft by a humoral mechanism.

INTRODUCTION

The recent availability of pigs homozygous for α1,3-galactosyltransferase gene-knockout (GT-KO) (1,2) has enabled pig-to-baboon organ transplantation to be carried out in the absence of expression of the pig Galα1,3Gal(Gal) epitopes that are known to be important targets for primate anti-pig antibodies (3-6). Transplantation of hearts (7) and kidneys (8) from GT-KO miniature swine into immunosuppressed baboons that expressed either no or very low levels of preformed antibodies pre-transplantation against antigens on GT-KO pig peripheral blood mononuclear cells (PBMC) ('nonGal' antigens) was followed by relatively prolonged graft survival, extending to 179 days (heart) and 83 days (kidney). Heart graft failure was largely a result of the development of a thrombotic microangiopathy that may be a form of delayed antibody-mediated rejection. Kidney grafts from GT-KO pigs transplanted into inadequately immunosuppressed baboons developed an elicited antibody response directed to nonGal antigens that resulted in rejection of the graft within 8 to 16 days (9).

It is clear, therefore, that anti-nonGal antibodies can be associated with rejection or injury of GT-KO pig organs in baboons. There have hitherto been no definitive reports on the incidence of anti-nonGal antibodies in humans nor of the extent of complement-dependent cytotoxicity (CDC) towards pig cells. We here report on the incidence and cytotoxicity of

preformed antibodies directed to nonGal antigens on GT-KO pig PBMC in healthy human sera.

There are several reports suggesting that patients previously sensitized to alloantigens may have antibodies that cross-react against pig cells (reviewed in 10), and therefore may be at a greater risk from antibody-mediated rejection following pig organ xenotransplantation. We have measured the incidence and cytotoxicity of antibodies directed to nonGal antigens on GT-KO pig PBMC in the sera of allosensitized patients awaiting kidney transplantation.

METHODS

Human Subjects

Serum was collected from 27 healthy volunteers of all ABO blood types who had no history suggesting previous exposure to pig antigens or to alloantigens (no pregnancy, blood transfusion, organ or cell allotransplant). A random six sera were tested by enzyme-linked immunosorbent assay (ELISA) for allosensitization (see below), and all were negative (ELISA-PRA<10%). The sera were used for the detection of anti-pig and anti-nonGal antibodies by flow cytometry, and CDC, using wild-type (WT) and GT-KO pig PBMC, respectively, as targets.

Serum was similarly tested from 31 patients awaiting renal transplantation. These sera were evaluated in the Tissue Typing Laboratory of the University of Pittsburgh Medical Center using commercial LAT ELISA kits (One Lambda, Canoga Park, CA), according to the manufacturer's instructions, to identify IgG anti-class I and/or class II HLA-specific alloantibodies. Briefly, the HLA antibody was measured by a second enzyme-linked colorimetric reaction, and was read at 630nm using an ELISA reader (ELX 800, Bio-Tek). All tests were duplicated. The percentage of patient ELISA versus. positive IgG control was determined, and sera were identified as either unsensitized (ELISA-PRA<10%, n=16) or allosensitized (ELISA-PRA>70%, n=15).

Pigs and Isolation of Porcine PBMC

PBMC were collected from WT and GT-KO pigs (both provided by Revivicor, Inc., Blacksburg, VA) (1), both of Large White/Landrace/Duroc cross-breed, and all of blood type O. Although the WT and GT-KO pigs were from the same genetic background, they were not from identical clones; however, the only major difference was in the absence of Gal antigens in the GT-KO pigs. All animal care procedures were in accordance with the *Principles of Laboratory Animal Care* formulated by the National Society for Medical Research and the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication No. 86-23, revised 1985).

PBMC from several different pigs (WT and GT-KO) were isolated from heparinized blood by centrifugation over Ficoll-Paque PLUS (Amersham Biosciences, Piscataway, NJ). The total mononuclear cell fraction was washed twice with RPMI 1640 medium (Gibco, Grand Island, NY) and then resuspended in FACS buffer (PBS containing 1%BSA and 0.1%NaN_3) for flow cytometry or in RPMI medium (including 10%FBS) for cytotoxicity assay.

Flow Cytometry

Human serum samples were incubated for 30min at 56˚C to inactivate complement. Isolated PBMC ($1x10^6$ cells/tube) were incubated with 20µL of serum (20% final concentration) or 20µL of FACS buffer (control) for 30min at 4˚C. (A 20% serum concentration was selected after an initial dilution study.) After incubation, the cells were washed twice in 2mL FACS buffer and centrifuged at 1800rpm for 5min. The supernatant was discarded. To prevent non-specific binding, 10µL of 10% goat serum was added. Detection of IgG or IgM binding was performed by further incubating the serum with FITC-conjugated goat anti-human IgG (γ chain-specific) at 1:50 dilution or IgM (µ chain-specific) at 1:200 dilution (Zymed, San Francisco, CA) for 30min in the dark at 4˚C. The samples were washed twice, and the cells resuspended with FACS buffer. Data acquisition was performed with FACScan (Becton Dickinson; Mountain View, CA). Dead cells were excluded from the analysis by forward-scatter and propidium iodide staining. IgG and IgM anti-pig antibodies were determined by indirect immunoflorescence intensity. Measurement of mean florescence intensity (MFI) was performed on viable cells. Measurement of indirect immunoflorescence intensity was accomplished by Cell Quest software and converted into MFI by WinMDI software.

CDC Assay

Human serum was incubated for 30min at 56˚C to inactivate complement. The samples were four-fold serially diluted with RPMI 1640 media (+10% FBS) to final concentrations of 50%, 12.5%, 3.125%, 0.78% and 25%, 6.3%, 1.6% and 0.4%. As an interplate control, serum from a single human was diluted in the same manner and used on all plates. Cells were loaded into flat-bottomed 96-well plates at a density of $1x10^6$ cells/well (in RPMI 1640 media + 10%FBS). The cells were centrifuged at 1800rpm for 7min, and then incubated with 100µL of diluted human (interplate control) serum for 30min at 4°C.

After incubation, the cells were incubated with 100µL of 10% rabbit HLA-ABC serum (Sigma), as a source of complement, for 45min at 37°C. Negative control wells (100% live cells) were achieved by adding only complement. Positive control wells (0% live cells) were achieved by adding a detergent (polyoxyethylene sorbitan monolaurate; Sigma). Additional negative controls comprised either cells alone or cells + serum in wells in which no complement or detergent was added.

Following incubation, the cells were resuspended in 100µL of RPMI 1640 (+10%FBS) and 20µL of tetrazolium compound (CellTiter 96® Aqueous One Solution Reagent; Promega,

Madison, WI) and incubated for 15h, for the development of color, at 37˚C in a 5%CO_2 humidified atmosphere. Cell viability was assessed by the capacity of live cells to reduce tetrazolium salts into formazan, and the color produced was measured (absorbance) using a plate reader (Bio-Tek Instruments, Winooski, VT) at a wavelength of 450nm. The assumption made was that a cell incapable of producing color is a dead cell.

Cell viability was calculated as:

$$\% \text{ live cells} = [(A-B)/(C-B)] \times 100,$$

where A equals the absorbance of cells (+ serum and complement), B equals the absorbance of cells (+ detergent), and C equals the absorbance of cells (+ complement only).

Results were expressed as the percentage of dead cells (% cytotoxicity = 100 - % viability). CDC values at the various serum concentrations were calculated, and a curve was generated. Lysis of PBMC of <10% was considered of doubtful relevance.

Statistical Analysis

The results were statistically analyzed by Mann-Whitney U tests or analysis of variance (ANOVA). A *p* value of <0.05 was considered statistically significant. Correlation of CDC and MFI was assessed by linear regression analysis. Significance at the 95% or the 99% level was calculated using Prism-4 software (Graphpad Software, San Diego, CA).

RESULTS

Reactivity of Serum from Healthy Humans to PBMC from WT and GT-KO Pigs

Binding of IgM and IgG to pig PBMC

All of the 27 sera (100%) showed some IgM (Figure 1A) and IgG (Figure 1B) binding to WT PBMC (% number indicates the percentage of sera with binding to PBMC that was greater than the mean control binding [secondary antibody alone] +SEM). In contrast, 21 of the 27 sera (78%) showed IgM binding, and only 17 showed IgG binding (63%) to GT-KO PBMC. Binding to GT-KO PBMC was detected in a total of 23 of the 27 sera (85%); 4 sera (15%) showed no detectable, or negligible, binding to GT-KO PBMC. The incidence of binding of IgM and IgG to GT-KO PBMC was therefore 78% and 63%, respectively, of that to WT PBMC.

The mean MFI of IgM binding to WT and GT-KO PBMC control (no serum) was 3.2 and 3.3, respectively (not significantly different, NS) (Figure 2A). The mean MFI of IgM binding to WT and GT-KO PBMC was 10.0 and 4.5, respectively ($p<0.05$). The mean MFI of IgG binding to WT and GT-KO PBMC control (no serum) was 3.2 and 3.3, respectively (NS) (Figure 2B). The mean MFI of IgG binding to WT and GT-KO PBMC was 12.4 and 6.2, respectively

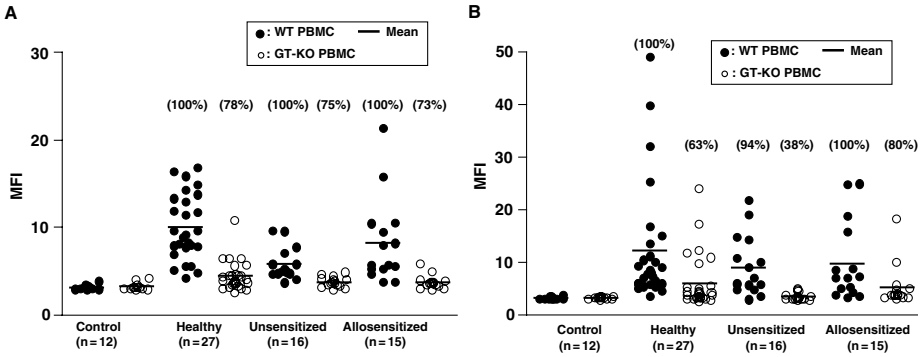

Figure 1:
Binding of xenoreactive antibodies in sera from healthy human volunteers and unsensitized or allosensitized patients to WT and GT-KO PBMC (MFI = mean fluorescence intensity). Distribution of serum reactivity of 27 healthy humans, 16 unsensitized, and 15 allosensitized humans against WT (●) or GT-KO (○) PBMC is shown - IgM **(A)** and IgG **(B)**. Mean reactivity of each group is indicated by a line. Control is FITC-conjugated antibody only. (% number indicates the percentage of sera with binding to PBMC that was greater than the mean control binding [secondary antibody alone] +SEM).

($p<0.05$). However, one serum showed a moderately high level of IgM binding (and of IgG binding and cytotoxicity), and 6 showed moderately high IgG binding to GT-KO PBMC (MFI 19.8 - 24.1) (Figure 1A and B); these sera also showed particularly high IgG binding to WT PBMC (MFI 10.4 - 49.0). (These 6 sera, however, did not show a consistently high cytotoxicity to GT-KO PBMC).

These results indicate that (i) the greater binding to WT PBMC was due to the presence of Gal-specific antibodies, and (ii) the residual binding to GT-KO PBMC was due to the presence of antibodies to nonGal antigens.

CDC of sera to pig PBMC

Of the 27 sera screened by flow cytometry, only 18 were available in sufficient quantity to allow CDC assay. At 50% serum dilution, all 18 sera (100%) were cytotoxic (>10% killing) to WT PBMC, whereas only 11 of the 18 (61%) showed some cytotoxicity to GT-KO PBMC (Figures 3A and B). Furthermore, the sera were significantly more cytotoxic to PBMC from WT than GT-KO pigs ($p<0.05$); at 50% dilution, the lysis of PBMC was reduced from 45% lysis (WT) to 19% (GT-KO) (Figure 3B). However, two sera showed relatively high cytotoxicity to GT-KO PBMC; these two sera did not have particularly high IgM or IgG binding to GT-KO PBMC. A serum dilution of 12.5% caused lysis of WT PBMC (mean lysis 31%), whereas GT-KO cells were generally largely resistant (mean lysis 10%) (Figure 3A).

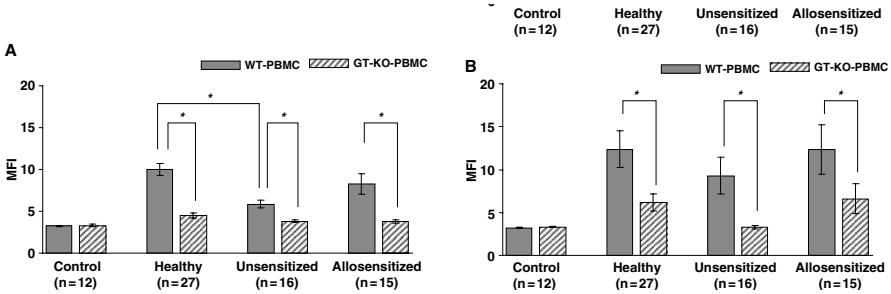

Figure 2:
Mean reactivity (MFI levels ± SEM) of sera from 27 healthy humans, 16 unsensitized, and 15 allosensitized patients against WT (grey bar) or GT-KO (shaded bar) PBMC - IgM **(A)** and IgG **(B)**. Statistically higher ($p<0.05$) reactivity of both IgM and IgG against WT PBMC versus GT-KO PBMC was observed, irrespective of the type of serum tested (*$p<0.05$). The MFI of IgM binding of unsensitized sera was significantly lower to WT PBMC than that of healthy sera.

We concluded that, whereas all healthy human sera demonstrate antibodies that are cytotoxic to WT pig PBMC, only approximately two-thirds of sera demonstrate anti-nonGal antibodies that are cytotoxic to PBMC from GT-KO pigs, and the level of cytotoxicity is generally relatively weak.

Reactivity of Serum from Allosensitized Humans to PBMC from WT and GT-KO Pigs

Binding of IgM and IgG to pig PBMC

Two sets of sera were available from patients awaiting kidney transplantation – patients who were (i) not allosensitized (PRA<10%, n=16; 'unsensitized'), and (ii) allosensitized (PRA>70%, n=15; 'allosensitized').

All unsensitized and allosensitized sera (100%) showed some detectable IgM binding to WT PBMC (Figure 1A). Twelve of the 16 unsensitized (75%) and 11 of the 15 (73%) allosensitized sera showed detectable IgM binding to GT-KO PBMC (Figure 1A). The mean MFI of IgM binding to WT and GT-KO PBMC was calculated to be 5.8 and 3.8, respectively, for unsensitized sera, and 8.3 and 3.8, respectively, for allosensitized sera (NS). Mean IgM binding of both unsensitized and allosensitized sera to WT PBMC was higher than to GT-KO PBMC ($p<0.05$) (Figure 2A).

Ninety four percent of unsensitized and 100% of allosensitized sera showed some detectable IgG binding to WT PBMC (Figure 1B). Six of the 16 (38%) unsensitized and 12 of the 15 (80%) allosensitized sera showed detectable IgG binding to GT-KO PBMC (Figure 1B). The mean MFI of IgG binding to WT and GT-KO PBMC was calculated to be 9.3 and 3.3,

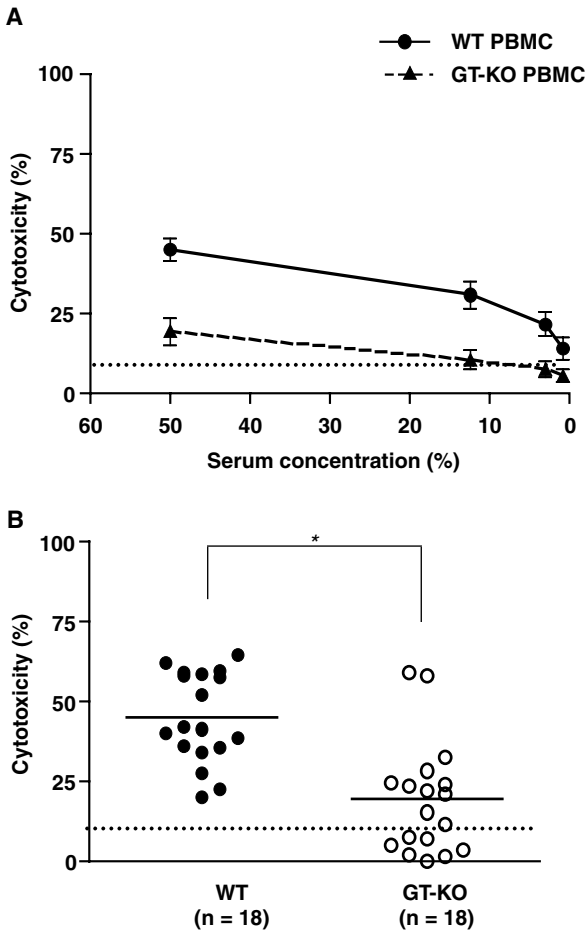

Figure 3:
(A) Linear regression of complement-dependent cytotoxicity of healthy sera (n=18) against WT (circle and solid line) and GT-KO (triangle and broken line) PBMC. The cytotoxicity of serum at various concentrations (50%, 12.5%, 3,125%, and 0.78%) was measured, and individual curves were generated. Significantly higher ($p<0.05$) lysis of WT PBMC was observed. **(B)** Ability of individual healthy serum samples (n=18) (at 50% dilution) to cause lysis of PBMC from WT (●) or GT-KO (○) pigs; mean complement-dependent cytotoxicity is indicated by horizontal lines. Significantly higher lysis of WT PBMC was observed ($p<0.05$). The horizontal dotted line indicates 10% cytotoxicity, below which lysis of PBMC is considered of doubtful relevance.

respectively, for unsensitized sera, and 12.3 and 6.6, respectively, for allosensitized sera (NS). Mean IgG binding of unsensitized and allosensitized sera to WT PBMC was higher than to GT-KO PBMC ($p<0.05$) (Figure 2B).

CDC of sera to pig PBMC

Thirteen unsensitized and 14 allosensitized sera were available for testing. (In addition, 12 healthy human sera were tested again.) At 25% dilution, all unsensitized and allosensitized sera showed cytotoxicity (>10% killing) to WT PBMC, whereas 10 of the 13 unsensitized (77%) and 7 of the 14 allosensitized (50%) sera were cytotoxic to GT-KO PBMC. The mean cytotoxicity (at 25% dilution) of unsensitized and allosensitized sera to WT PBMC was 53% and 60%, respectively, and to GT-KO PBMC was 16% and 18%, respectively (Figure 4).

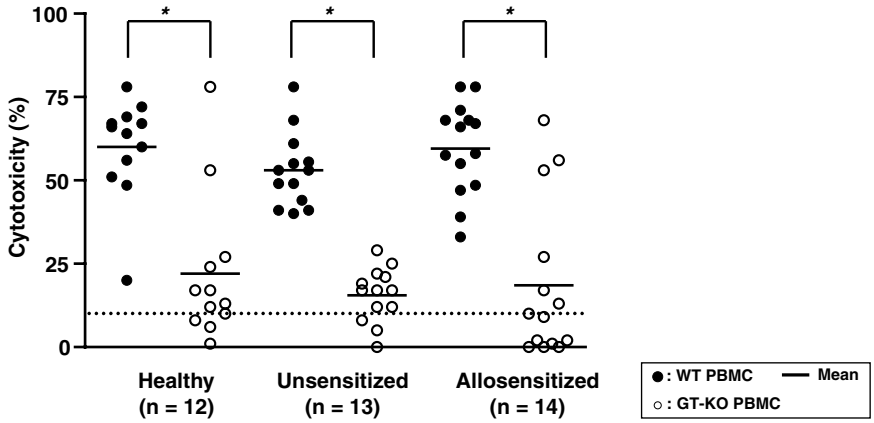

Figure 4:
Ability of healthy (n = 12), unsensitized (n=13), and allosensitized (n=14) sera (at 25% dilution) to cause lysis of PBMC from WT (●) or GT-KO (○) pigs; mean complement-dependent cytotoxicity is indicated by horizontal lines. Significantly higher lysis of WT PBMC was observed (*$p<0.05$). The horizontal dotted line indicates 10% cytotoxicity, below which lysis of PBMC is considered of doubtful relevance. There was no difference in cytotoxicity to either WT or GT-KO PBMC between the three groups of sera. (A serum dilution of 25% was selected as the volumes of allosensitized sera were limited).

Correlation between PRA against HLA Class I or Class II Antigens and Serum Cytotoxicity in Human Sera

We investigated whether there was any correlation between serum cytotoxicity to pig PBMC and anti-HLA class I or class II antibodies (at 25% dilution) (n=27; 13 unsensitized and 14 allosensitized sera). No correlation was found between PRA directed to HLA class I or class II and cytotoxicity to WT or GT-KO PBMC (Figure 5).

Comparison of Binding of IgM and IgG and Serum Cytotoxicity between Healthy and Allosensitized Sera

The mean MFI of IgM binding of unsensitized sera to WT PBMC was significantly lower than that of healthy sera ($p<0.05$) (Figure 2A). There was no difference in IgM binding to WT or GT-KO PBMC between allosensitized sera and healthy or unsensitized sera. There was no difference in IgG binding to WT or GT-KO PBMC between healthy, unsensitized, or allosensitized sera (Figure 2B). There were no significant differences in cytotoxicity between healthy, unsensitized, and allosensitized sera to WT PBMC (Figure 4A) or to GT-KO PBMC (Figure 4B).

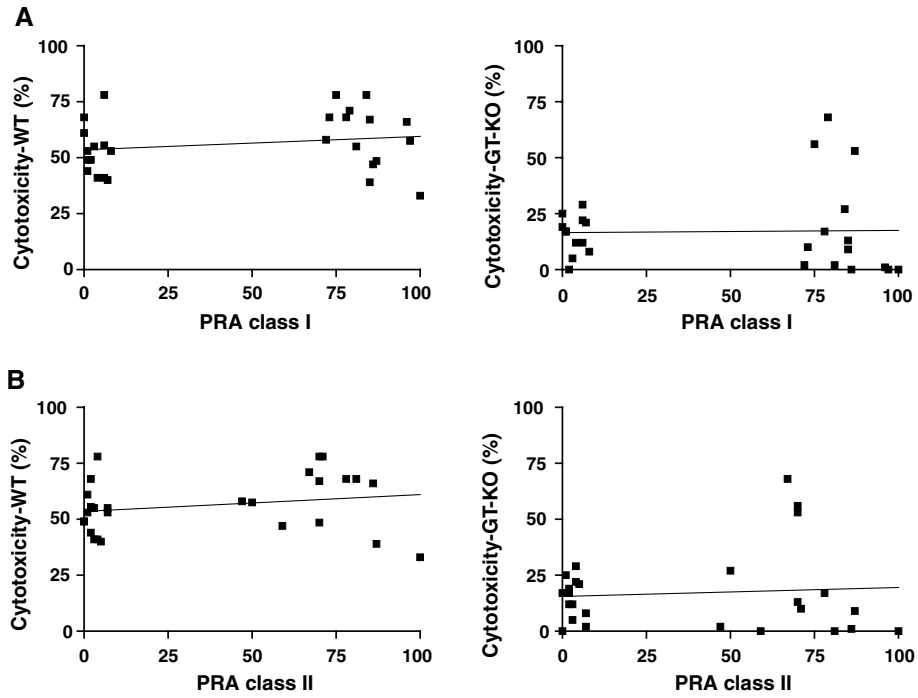

Figure 5:
Linear regression of cytotoxicity (at 25% serum concentration) of unsensitized and allosensitized sera against PRA to HLA class I **(A)** or HLA class II **(B)**. Correlation is shown for cytotoxicity to WT (left) and GT-KO (right) PBMC (n=27). There was no significant correlation between PRA against HLA class I or II and cytotoxicity to either WT or GT-KO PBMC.

Correlation between Immunoglobulin Binding and Cytotoxicity in Sera from All Groups

In the absence of any relevant difference in immunoglobulin binding or cytotoxicity between healthy, unsensitized, or allosensitized sera, we investigated the correlation of immunoglobulin isotype with cytotoxicity in 39 sera available for testing (12 healthy, 13 unsensitized, 14 allosensitized). A correlation was found between IgM binding and cytotoxicity (at 25% serum concentration) to WT PBMC (R^2=0.13, $p<0.05$) (Figure 6A, left), but not between IgG binding and cytotoxicity to WT PBMC (R^2= 0.07, $p>0.05$) (Figure 6B, left). In contrast, correlations between both IgM and IgG binding and cytotoxicity to GT-KO PBMC were documented (R^2= 0.27, $p<0.01$, and R^2= 0.28, $p<0.01$, respectively) (Figures 6A and B, right). The level of lysis of GT-KO PBMC was generally lower than that of WT PBMC, even when the MFI of binding was identical.

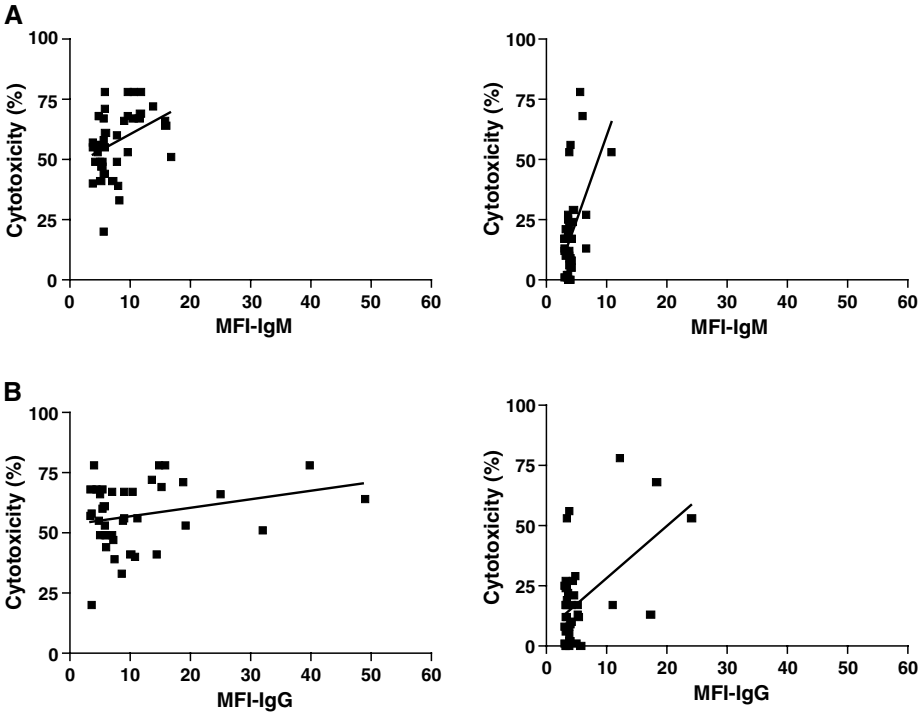

Figure 6:
Correlation between cytotoxicity (at 25% serum concentration) and IgM **(A)** or IgG **(B)** binding of 39 sera (12 healthy, 13 unsensitized, 14 allosensitized) against WT PBMC (left) or GT-KO PBMC (right). There was significant correlation between IgM binding and cytotoxicity against WT PBMC ($p<0.05$), but not between IgG binding and cytotoxicity. Significant correlations were observed between IgM or IgG binding and cytotoxicity (both $p<0.01$) to GT-KO PBMC

DISCUSSION

Organ transplantation from a WT pig into a nonimmunosuppressed primate usually results in hyperacute rejection within minutes to hours (11,12). The primary role of anti-Gal antibodies in this response has been established by several groups (3-6). The problem of anti-Gal antibody has recently been overcome by the breeding of pigs homozygous for GT-KO (1,2). However, although the transplantation of an organ from a GT-KO pig into an immunosuppressed nonhuman primate allows extended graft survival (7), the graft eventually fails from the development of a thrombotic microangiopathy that may be a form of delayed antibody-mediated rejection. The focal deposition of IgM, IgG, and C4d on the graft suggests that a component of this process involves the binding of anti-nonGal antibodies to the pig vascular endothelial cells. These antibodies could be either preformed or elicited.

The present study investigated the incidence of preformed anti-nonGal antibodies in the serum of healthy and allosensitized humans, and assessed whether these antibodies are cytotoxic to pig PBMC lacking the Gal epitope. The results were compared with reactivity of anti-pig antibodies to WT pig PBMC.

Whereas all healthy and allosensitized humans had preformed antibodies to WT pig PBMC, in approximately one-third such antibodies to GT-KO PBMC were not detectable. Moreover, the cytotoxicity associated with anti-nonGal antibodies was significantly weaker than that associated with anti-Gal antibodies. Whereas reactivity with WT pig cells is clearly due to the binding of both anti-Gal and anti-nonGal antibodies, reactivity with GT-KO cells is solely due to the presence of anti-nonGal antibodies.

Healthy humans who demonstrated a high MFI of IgG binding to GT-KO PBMC also had a high IgG binding to WT PBMC, but did *not* have a consistently high serum cytotoxicity to GT-KO PBMC. These subjects would, therefore, not necessarily appear to be at greater risk for hyperacute rejection if they were to receive a GT-KO pig organ. The one healthy subject who had high IgM binding to GT-KO PBMC (Figure 1A) also had high IgG binding *and* cytotoxicity, and so would appear to be at higher risk.

Antibody binding, particularly of the IgM isotype, results in complement fixation and subsequent cell lysis. This was investigated by a two-step CDC assay. GT-KO pig PBMC were significantly less susceptible to lysis by either healthy or allosensitized sera than WT PBMC. However, although the absence of the Gal epitope led to a significant reduction in both the prevalence and severity of complement-induced lysis, anti-nonGal antibodies nonetheless can initiate a cytotoxic response which, *in vivo,* could affect long-term viability of a pig organ graft.

One possible limitation of our study is that only a random six sera of the 27 healthy human volunteers from whom sera were obtained were tested by ELISA for allosensitization. However, none of the volunteers had been pregnant or had received blood transfusions or organ allografts. It is therefore extremely unlikely that any were allosensitized.

A second possible limitation is that only PBMC were used as target cells, whereas vascular endothelial cells might have provided different, or more biologically relevant (for vascularized organs), nonGal epitopes, although there is no definite evidence for this (13). Chen et al. reported that baboon anti-nonGal antibody binding (both IgM and IgG) to GT-KO pig porcine aortic endothelial cells was similar to that to PBMC (14). We are currently investigating whether there are differences between anti-pig antibody binding to PBMC and vascular endothelial cells; preliminary results suggest that binding to PBMC may actually be higher (Hara, H, unpublished).

In sera from unsensitized patients, the mean MFI of IgM binding to WT PBMC was significantly lower than that in healthy human sera (Figure 2A), although the extent of IgG binding was not different (Figure 2B). We cannot fully explain this observation. One possible explanation is that patients in chronic renal failure are likely to be modestly immunocompromised. However, the allosensitized patients would also be expected to be immunocompromised, but IgM binding of their sera was not different from that of healthy volunteers. It could also be that unsensitized patients are 'low responders', but this reasoning is undermined by the observation that their IgG responses were not different from the other two groups. Furthermore, there was no difference in serum cytotoxicity between the three groups (Figure 4).

An important finding from this study is the documentation that sera from allosensitized subjects did not show a higher level of binding of preformed antibodies (IgM or IgG) to nonGal antigens than did sera from unsensitized or healthy subjects, nor increased cytotoxicity. Previously, some reports suggested that sera from allosensitized subjects might be more cytotoxic to unmodified pig cells than sera from unsensitized subjects, although other reports did not confirm this observation (reviewed in 10). If the data in the current paper are confirmed – that humans previously exposed and sensitized to alloantigens are at no greater risk for rejecting a GT-KO pig xenograft by an antibody-mediated mechanism, this will have important clinical implications. Highly allosensitized patients frequently have difficulty in obtaining a human donor organ against which they do not have anti-HLA antibodies. This group of patients may therefore be among those first considered for xenotransplantation.

Previous studies by others have suggested that anti-HLA antibodies can cross-react with anti-swine leukocyte antigens (SLA) (reviewed in 10). In the present study, we could not identify any correlation between the presence of anti-HLA antibodies and IgM or IgG binding or cytotoxicity to WT or GT-KO pig PBMC. The possibility of cross-reactivity between anti-HLA and anti-SLA antibodies cannot be completely ruled out as the SLA types of the pigs from which PBMC were obtained have not been determined.

The significant correlation found between IgM binding and cytotoxicity of sera to WT PBMC confirms previous studies (15,16), and supports the fact that xenoreactive IgM is more capable of fixing complement, whereas only some isotypes of IgG, and IgA, can fix complement (17-19). IgG binding to WT PBMC might have correlated with cytotoxicity if a greater number of sera had been available for study. Although significant correlations between IgM or IgG binding and cytotoxicity to GT-KO PBMC were also observed, the level of lysis was generally lower than that of WT PBMC even when the MFI of binding was identical (Figure 6).

Our data suggest that GT-KO pigs are more suitable than WT pigs as sources of organs for transplantation into humans, since reactivity with preformed antibodies in human serum was significantly reduced. The possibility that a second Gal-sythesizing enzyme in pigs,

e.g., iGb3 synthase (20), may be playing a role in rejection of a GT-KO pig organ appears unlikely since no changes in anti-Gal antibody levels have been documented after GT-KO pig heart transplantation in baboons, even when elicited antibodies have developed (7,21,22). However, unidentified nonGal epitopes on the pig vascular endothelium could still pose a threat to the viability of a porcine organ transplanted into a human. With the possible exception of N-glycolylneuraminic acid (23), the nature of the nonGal antigens expressed on the GT-KO pig vascular endothelium or PBMCs against which humans have *preformed* antibodies is uncertain, and has been discussed previously (24,25).

If there are multiple nonGal epitopes against which humans have preformed antibodies, then gene-knockout may not be a feasible solution. However, the lower level of endothelial cell activation and cytotoxicity associated with anti-nonGal antibodies, compared with anti-Gal antibodies, may allow protection by the presence of one or more human complement-regulatory proteins when combined with a GT-KO pig genotype. Although transgenic pigs expressing human decay-accelerating factor or other complement-regulatory protein were incompletely protected against anti-Gal antibodies (26,27), this approach may prove more successful against the weaker anti-nonGal antibodies.

There is increasing success in achieving a state of accommodation after allografting across the ABO blood group barrier or when there is allosensitization associated with anti-HLA antibodies (28). Such approaches, involving procedures such as pre- and post-transplant plasmapheresis and the administration of intravenous immunoglobulin (IVIg) (29), although not effective in inducing accommodation to a WT pig xenograft (30-32), may possibly prove more successful in the presence of the lower levels of anti-nonGal antibody. To our knowledge, this approach has not yet been tested in the GT-KO pig-to-nonhuman primate model.

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Chapter 5
Incidence and cytotoxicity of antibodies in cynomolgus monkeys directed to nonGal antigens, and their relevance for experimental models

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ABSTRACT

The recent availability of pigs homozygous for α1,3-galactosyltransferase gene-knockout (GT-KO) has enabled study of incidence and cytotoxicity of antibodies of cynomolgus monkeys directed to antigens other than Galα1,3Gal (Gal), termed nonGal antigens. To this aim, sera from 21 cynomolgus monkeys were tested by flow cytometry for binding of IgM and IgG to peripheral blood mononuclear cells (PBMC) from wild-type (WT) and GT-KO pigs. The sera were also tested for complement-dependent cytotoxicity to WT and GT-KO PBMC. Anti-WT IgM and IgG were found in 100% and 95%, respectively, and anti-GT-KO IgM and IgG in 76% and 66%, respectively, in the sera of the monkeys tested ($p<0.01$). Whereas 100% of sera were cytotoxic to WT PBMC, only 76% were cytotoxic to GT-KO PBMC, and the level of cytotoxicity was significantly less ($p<0.01$). Although the incidence and cytotoxicity of antibodies in monkey sera to GT-KO pig PBMC are significantly less than to WT PBMC, approximately three-quarters of the monkeys tested had cytotoxic antibodies to GT-KO PBMC. This incidence of cytotoxicity is significantly higher than that found in baboons and humans, suggesting the baboon may be an easier and possibly more suitable model to study antibody-mediated rejection of transplanted GT-KO pig organs and cells.

INTRODUCTION

The recent availability of pigs homozygous for α1,3-galactosyltransferase gene-knockout (GT-KO) (1, 2) has enabled pig-to-nonhuman primate organ and cell transplantation to be carried out in the absence of expression of the pig Galα1,3Gal (Gal) epitopes that are known to be important targets for primate anti-pig antibodies (3-6). Hyperacute and acute humoral xenograft rejection (AHXR) are largely caused by these anti-Gal antibodies. AHXR is believed to be a consequence of antibody binding to vascular endothelial cells, whereby anti-Gal as well as anti-nonGal antibodies (i.e., antibodies against targets other than Gal antigens) in the recipient are of importance. A varying incidence of anti-nonGal antibodies has been reported humans by Zhu (6-8). These anti-nonGal antibodies appear to be associated with rejection or injury of pig organs or cells in non-human primates (9, 10, and Ezzelarab, M., submitted for publication).

In experimental settings, macaque monkeys (i.e., cynomolgus and rhesus monkeys) and baboons are frequently used as recipients to study the survival of pig organs and cells. Possible differences in anti-nonGal antibodies between species are of importance as they may have implications for suitability of the preclinical model.

There have hitherto been no definitive reports on the incidence of anti-nonGal antibodies in cynomolgus monkeys, nor of the extent of cytotoxicity associated with these antibodies towards pig cells. We here report on the incidence and cytotoxicity of preformed antibodies

directed to nonGal antigens on GT-KO pig peripheral blood mononuclear cells (PBMC) in immunologically-naïve (not intentionally sensitized) cynomolgus monkeys. We also compare the incidence of anti-nonGal antibodies and their cytotoxicity in cynomolgus monkeys with results obtained by our group in baboons and humans.

METHODS

Animals

Serum samples were prepared from blood collected from 21 immunologically-naïve cynomolgus monkeys (*Macaca fascicularis)* of all blood groups (A, B, AB, O), purchased from different suppliers. To detect the presence of anti-pig and anti-nonGal antibodies in these samples, PBMC were collected from GT-KO and unmodified (wild-type, WT) pigs, and used as targets in flow cytometry and cytotoxicity assays. The pigs were obtained from Revivicor, Inc. (Blacksburg,VA) and were of Large White/Landrace/Duroc cross-breed (1); the GT-KO pigs differed from the WT pigs only with regard to the absence of Gal epitopes, which was confirmed by several different methods (1).

All animal care procedures were in accordance with the *Principles of Laboratory Animal Care* formulated by the National Society for Medical Research and the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication No. 86-23, revised 1985).

Isolation of Porcine PBMC

PBMC from several different pigs (WT and GT-KO) were isolated from heparinized blood; the PBMC were not pooled, but PBMC from a single pig were used for testing of several sera. Briefly, heparinized blood samples were subjected to gravity centrifugation at 1800rpm for 5 min at 20˚C. Plasma from each sample was then removed, and the remaining cells were resuspended in phosphate buffered saline (PBS, Sigma, St. Louis, MO). The final dilution of blood to PBS was 2:1. These cells were then layered slowly and carefully over 5mL Ficoll-Paque PLUS (Amersham Biosciences, Piscataway, NJ) in 15mL Falcon conical tubes (Becton Dickinson, Franklin Lake, NJ). The cells were gravity centrifuged at 1500rpm for 30 min at 20˚C, and the buffy coat containing the PBMC was isolated and collected in a separate tube. The total mononuclear cell fraction was then washed twice, filtered through a mesh and resuspended with RPMI 1640 media (Gibco, Grand Island, NY), and live cells were counted using trypan blue (Gibco) under light microscopy.

Flow Cytometry

Isolated PBMC were resuspended in FACS-buffer (PBS containing 1% bovine serum albumin [Gibco] and 0.1% NaN_3) to a final cell concentration of $12.5x10^6$ cells/mL. One million PBMC were then removed and incubated with 20µL of heat-inactivated monkey serum (at 56°C for 30 min), diluted in FACS buffer to 20% serum final concentration, for 30 min at 4˚C. After incubation, the cells were washed twice in 2mL FACS buffer, vortexed and centrifuged at 1800rpm for 5 min. Cells were then resuspended in 10µL of 10% goat serum in FACS buffer, to prevent non-specific binding, and incubated further with 10µL FITC-conjugated goat anti-human IgG (γ chain-specific) at 1:50 dilution, or with IgM (µ chain-specific) at 1:200 dilution, (Zymed Laboratories, San Francisco, CA) for 30 min in the dark at 4˚C. Antibodies bound to live cells were analyzed using FACScan (Becton Dickinson, Mountain View, CA). Dead cells were excluded from the analysis by forward-scatter and propidium iodide staining (10µL added to each sample prior to FACS analysis). Binding of a serum sample was considered positive when the mean fluorescence intensity (MFI) was higher than the MFI of the control (cells and secondary antibody alone) plus two standard deviations of the mean control level.

Complement-Dependent Cytotoxicity Assay

Heat-inactivated monkey serum samples (n=21) were diluted four-fold in RPMI 1640 media (+10% FBS; Gibco) to final concentrations of 50%, 12.5%, 3.125%, and 0.78%. As an interplate control, serum from a single human was diluted in the same manner and its complement-dependent cytotoxicity was tested on all plates. Cells were loaded into 96-well flat bottom plates at a cell density of $1x10^6$ cells per well in 10% FBS in RPMI 1640 media. The plates were centrifuged at 1800rpm for 7 min, the supernatant discarded, and the cells were incubated with 100µL of diluted monkey or human serum (control) for 30 min at 4°C. Control wells, no serum added, were loaded with 100µL of medium only. After incubation, the cells were washed (200µL RPMI 1640 +10% FBS), centrifuged at 1800rpm for 7 min, the supernatant discarded, and further incubated with 100µL of 10% rabbit HLA-ABC serum (Sigma), as a source of complement, for 45 min at 37°C. Negative control wells (100% live cells) were those to which only complement was added; positive control wells (0% live cells) were those in which a detergent (polyoxyethylene sorbitan monolaurate; Sigma) was added to lyse all cells. Additional negative controls were employed; these comprised either of cells alone or cells and serum.

Following incubation, the cells were washed and resuspended in 100µL of RPMI 1640 (+10% FBS) and 20µL of MTS (CellTiter 96® Aqueous One Solution Reagent; Promega, Madison, WI) and incubated for 15 h, for the development of color, at 37˚C in a 5% CO_2 humidified atmosphere. Cell viability was assessed by the capacity of live cells to reduce tetrazolium salts into formazan, and the color produced was measured (absorbance) using a plate reader (Bio-Tek Instruments, Winooski, VT) at a wavelength of 450nm. The assumption made in this assay was that a cell that is incapable of producing color is a dead cell. Cell

viability was calculated using the following formula:

$$\% \text{ live cells} = [(A-B)/(C-B)] \times 100,$$

where A equals the absorbance of cells (+ serum and complement), B equals the absorbance of cells (+ detergent), and C equals the absorbance of cells (+ complement).

Results were expressed as the percentage of dead cells (% cytotoxicity = 100 - % viability). Complement-dependent cytotoxicity values of the varying serum concentrations (50%, 12.5%, 3.125%, and 0.78%) were calculated, and a curve was generated for each monkey sample. Lysis of PBMC of <10% was considered of doubtful relevance.

Data Analysis

Analysis of indirect immunofluorescence intensity was accomplished by Cell Quest software and converted into MFI by WinMDI software. Statistical analysis of data was performed using Mann-Whitney U tests. Correlation of cytotoxicity and MFI was assessed by linear regression analysis. Significance at the 95% or the 99% level was calculated using prism-4 software (Graphpad Software, San Diego, CA).

RESULTS

Reactivity of Cynomolgus Monkey Serum Samples to PBMC from WT and GT-KO Pigs

Binding of monkey IgM and IgG to pig PBMC by flow cytometry

The presence of preformed xenoreactive antibodies in sera from immunologically naïve (not intentionally sensitized) cynomologus monkeys was assessed by flow cytometry. All 21 serum samples had a detectable IgM binding to WT PBMC, and 16 of 21 sera (76%) showed some binding to PBMC from GT-KO pigs (Figure 1A). A similar finding was observed with IgG binding; 20 of 21 samples (95%) demonstrated IgG binding to WT PBMC, while only 14 (67%) bound GT-KO cells (Figure 1A). In other words, with the flow cytometry assay employed in these studies, the incidence of binding of monkey IgM and IgG antibodies to GT-KO PBMC was 76% and 70%, respectively, of that to WT PBMC.

The mean MFI of IgM binding to WT PBMC and to GT-KO PBMC was determined to be 12.14 ± 3.54 and 6.66 ± 2.45, respectively; this mean MFI level of IgM binding to WT PBMC was significantly higher than that to GT-KO PBMC ($p < 0.01$, Mann-Whitney U) (Figure 1B). The mean MFI of IgG binding to WT PBMC was 9.96 ± 6.34, and the mean binding of IgG to GT-KO PBMC was 4.30 ± 1.17, the difference also achieving statistical significance ($p<0.01$) (Figure 1B).

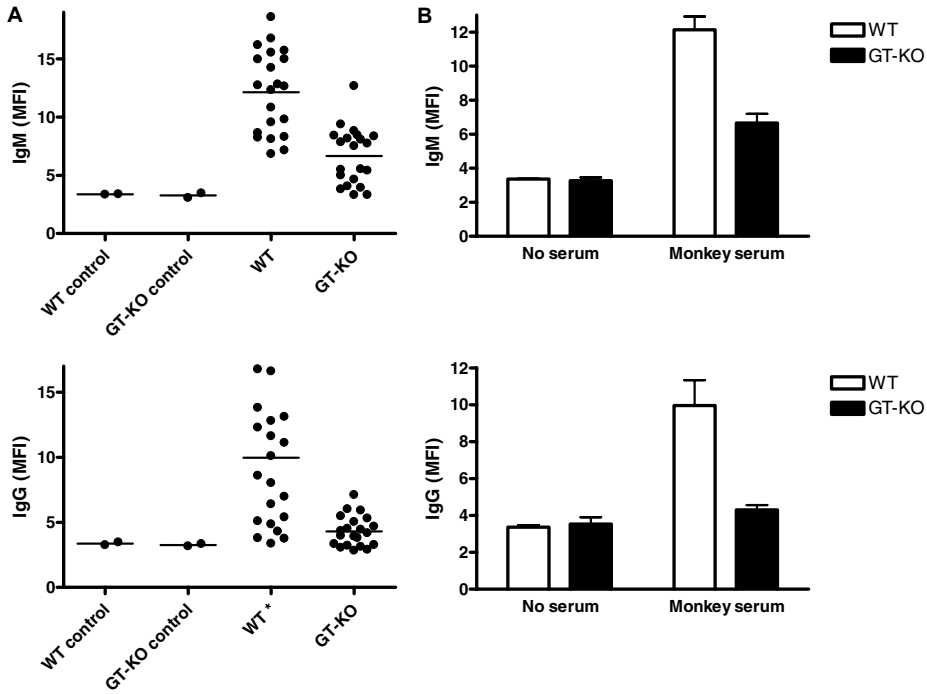

Figure 1:
Binding of cynomolgus monkey serum antibodies to WT and GT-KO pig cells (MFI = mean fluorescence intensity).
(A) Distribution of serum reactivity of 21 monkeys against WT or GT-KO PBMC, IgM (top) and IgG (bottom). Mean reactivity of each group is indicated by a line. Control is FITC-conjugated antibody and pig cells only.
(B) Mean reactivity (MFI levels ± SEM) of serum samples (n=21) against WT or GT-KO PBMC, IgM (top) and IgG (bottom). Statistically higher reactivity of both IgM and IgG against WT PBMC versus GT-KO PBMC was observed ($p<0.01$). No significant difference was found between IgM and IgG binding to GT-KO PBMC and GT-KO control.

These results would suggest that (i) the greater binding to WT PBMC was due to Gal-specific antibodies, and (ii) the residual binding to GT-KO PBMC was due to the presence of antibodies to nonGal-specific antigens.

Complement-dependent cytotoxicity of monkey sera to pig PBMC

After establishing that monkey serum can contain anti-nonGal antibodies, it was important to determine whether these antibodies can cause lysis of cells from GT-KO pigs. Lysis was assessed by using a classical two-step complement-dependent cytotoxicity assay. The cytotoxicity of the monkey serum samples was tested at various concentrations (50%,

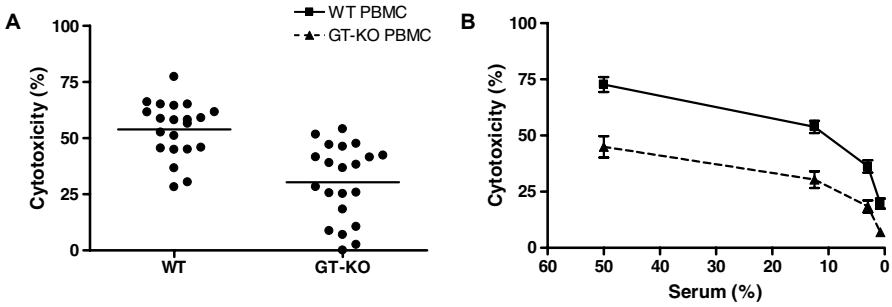

Figure 2:
(A) Ability of individual monkey serum samples at 12.5% dilution to cause lysis of PBMC from WT or GT-KO pigs; mean complement-dependent cytotoxicity is indicated by lines. Significantly higher lysis of unmodified (WT) PBMC was observed ($p < 0.01$).
(B) Mean cytotoxicity of monkey serum samples (at various dilutions) against PBMC from either WT or GT-KO pigs.

12.5%, 3.125%, and 0.78%). In three cases there was insufficient serum to include a 50% concentration in the assay.
The result of the assay was considered acceptable when the slope of the human control curve was within the 95% confidence limit of the mean of >25 determinations. Cytotoxicity results from 19 out of 21 sera fell within this confidence interval; however, there were no statistically significant differences whether 19 or 21 sera were included in the data analysis. (The sensitivity of the assay was such that only lysis of >10% was considered positive.)

The strength of serum cytotoxicity to both WT and GT-KO PBMC varied between the different monkeys. At 12.5% dilution, all the sera were cytotoxic to WT PBMC, whereas only 76% of these samples were lytic to PBMC from GT-KO pigs (Figure 2A). Furthermore these sera caused greater lysis of PBMC from WT pigs than from GT-KO pigs ($p<0.01$) (Figures 2A and B); at 12.5% dilution, the lysis of PBMC was reduced from approximately 54% (WT) to 30% (GT-KO) (Figure 2B).

We concluded that approximately three quarters of naïve monkey sera tested in this study contained anti-nonGal antibodies that are cytotoxic to PBMC from GT-KO pigs, though the extent of cell lysis is generally less than to WT pig PBMC.

Correlation between binding of IgM or IgG and serum cytotoxicity
To investigate if one of the immunoglobulin isotypes was more associated with cytotoxicity, correlation between cytotoxicity and binding (MFI) was assessed. Correlations were found between IgM binding to both WT and GT-KO PBMC and cytotoxicity (at 12.5% serum

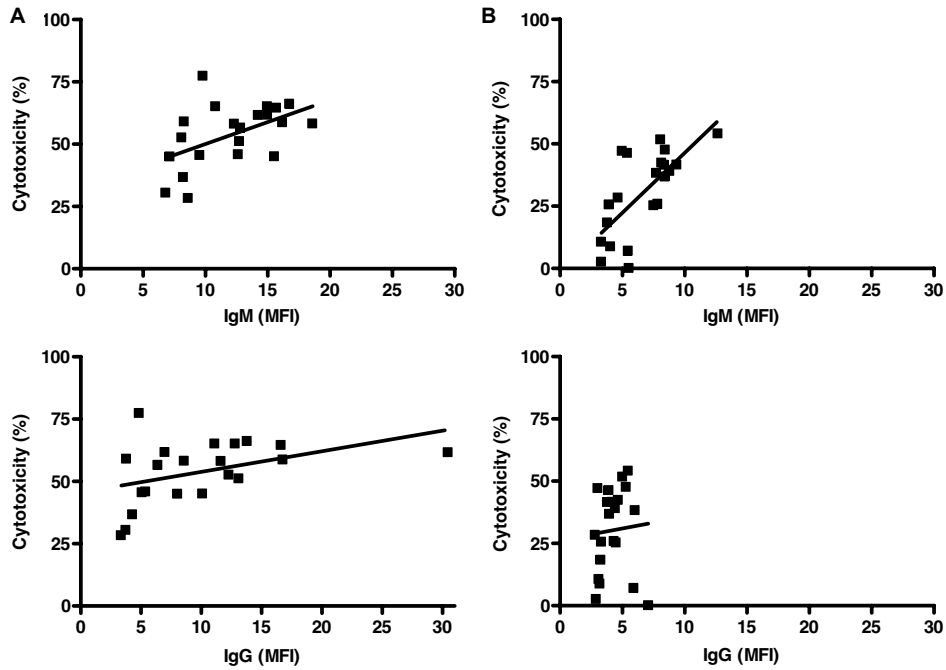

Figure 3:
Linear regression of monkey serum cytotoxicity (at 12.5% serum concentration) and immunoglobulin isotype binding to **(A)** WT and **(B)** GT-KO PBMC (n=21). Correlations were found between IgM binding (top) to both WT and GT-KO PBMC and cytotoxicity; no statistically significant correlation was observed between IgG binding (bottom) to WT and GT-KO PBMC and cytotoxicity.

concentration) ($p<0.02$) (Figure 3). However, no statistically significant correlation between IgG binding and cytotoxicity to WT or GT-KO PBMC was observed (Figure 3).

DISCUSSION

The incidence of binding of monkey serum IgM and IgG isotypes to PBMC from GT-KO pigs was significantly less when compared to binding to WT PBMC. However, we demonstrated that 76% of the tested monkey sera caused lysis of GT-KO PBMC (compared to 100% lysis of WT PBMC), although, the extent of cell lysis associated with anti-nonGal antibodies was significantly less. Even though this confirms the potential advantage of using GT-KO donor pigs for transplantation into cynomolgus monkeys, nevertheless approximately three quarters of the monkeys had IgM with or without IgG binding to GT-KO PBMC; as these

anti-nonGal antibodies are cytotoxic, they are likely to be associated with the development of AHXR.

One possible limitation of our study is that only PBMC were used as target cells, whereas vascular endothelial cells might have provided different, or more biologically relevant, nonGal epitopes, although there is no definite evidence for this. We are currently investigating whether there are differences between anti-pig antibody binding and cytotoxicity to PBMC and vascular endothelial cells; preliminary results suggest that there is little difference and, indeed, binding to PBMC may be higher (Hara H, et al, unpublished data). Furthermore, although PBMC were drawn from WT pigs from the same herd and from GT-KO pigs from three closely-related clones, neither WT nor GT-KO PBMC were pooled; the possibility of variable Gal and nonGal epitope expression between the PBMC cannot be ruled out, although this is likely to have been minor.

In other studies by our group, 43% of human (preliminary data, Hara H.) and 32% of baboon (Ezzelarab M, et al, manuscript submitted) sera were demonstrated to have IgM binding to GT-KO PBMC, which is significantly less than the 76% IgM binding of cynomolgus monkey sera that we report here ($p<0.01$). Both human and monkey sera showed an incidence of 67% IgG binding to GT-KO PBMC, compared to an incidence of only 9% of baboon IgG ($p<0.01$). The levels of IgG and IgM binding of all three species are shown in Figure 4. Furthermore, whereas approximately 50% of both human and baboon sera demonstrated cytotoxic antibodies against GT-KO PBMC, the incidence is 76% in monkey sera ($p<0.01$)

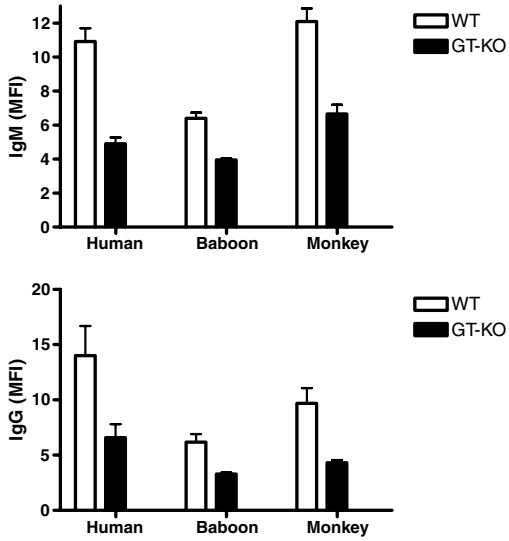

Figure 4:
Comparison of binding of human, baboon and cynomolgus monkey preformed xenoreactive antibodies to WT and GT-KO pig PBMC. Mean reactivities of human (n=21), baboon (n=56), and monkey (n=21) sera against WT or GT-KO PBMC are shown, IgM (top) and IgG (bottom).

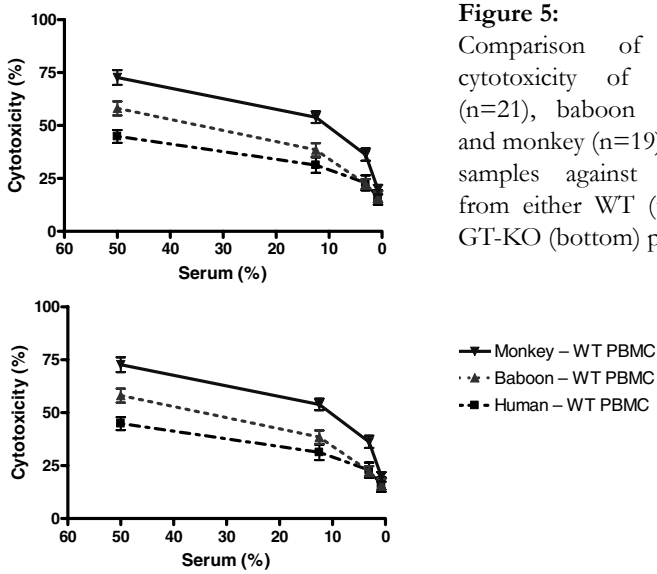

Figure 5:
Comparison of mean cytotoxicity of human (n=21), baboon (n=39), and monkey (n=19) serum samples against PBMC from either WT (top) or GT-KO (bottom) pigs.

(Figure 5). These results suggest that monkeys may have stronger antibody-mediated cytotoxic responses to GT-KO pig grafts than humans and baboons.

In experimental settings, GT-KO organs have been transplanted into immunsuppressed baboons; heterotopic heart graft failure occurred after 2-6 months (11) and recipients with life-supporting pig kidneys died from complications of the regimen within 3 months, although in some transplant kidney function continued (12). Whereas anti-Gal antibodies could not have been part of this rejection process, anti-nonGal antibodies could possibly have been involved. These findings are supported by a study from Brandl et al. in an hDAF transgenic pig-to-baboon heart transplantation model using soluble Gal saccharides to prevent anti-Gal antibody-mediated hyperacute rejection; this center reported that nonGal antibody titers were increased, and this increase was associated with the development of AHXR (9).

To our knowledge, there are no reports of transplantation of GT-KO organs or cells in cynomolgus monkeys. However, there is evidence that anti-nonGal antibodies are associated with AHXR in cynomolgus monkeys; Lam et al. demonstrated that, when hDAF-transgenic pig hearts were transplanted into cynomolgus monkeys under soluble Gal saccharide-based therapy (to adsorb or 'neutralize' anti-Gal antibodies), anti-nonGal antibody levels increased in animals that rejected their grafts (10).

Although the identity of pig nonGal antigens still remains uncertain (13), there is evidence that anti-N-glycolylneuraminic acid antibodies, also known as Hanganutziu-Deicher (HD)

antibodies, are present in human serum (8, 14). Since pigs express N-glycolylneuraminic acid on their cells, this may, in part, account for human anti-nonGal antibody binding to GT-KO PBMC. However, since baboons and monkeys express HD antigens themselves (like the pig), anti-HD antibodies do not contribute to the anti-nonGal antibodies found in these nonhuman primates species. The higher level of anti-nonGal IgM in monkeys detected in our studies compared to that in humans and baboons cannot be fully explained, but it is probably related to differences in the microbial flora that populate the gastro-intestinal tract or to other infectious agents to which these various species have been exposed. Also the age, geographic origin, and whether wild-caught or captive-bred, of the monkeys from which sera were drawn may have played a role in this (15-17), but unfortunately this information was not available to us for sufficient monkeys to draw conclusions in this respect. The target(s) for these antibodies needs to be ascertained.

The observed differences in the levels of anti-nonGal IgM and IgG and cytotoxicity between the species may be, at least in part, related to the relatively small numbers in each group. A study involving sera from larger numbers of each species may find less variation. A factor that must also be considered is the isotypes of anti-nonGal IgG in the three species, which we did not explore. Although not identified in this study, the ability of the IgG isotype to activate complement may have been an important factor in the correlation between antibody and cytotoxicity. However, if monkeys are confirmed to have higher levels of anti-nonGal IgM, this may be associated with a higher incidence of hyperacute rejection of a transplanted pig organ than in either baboons or humans.

In addition to this potential role in the rejection of GT-KO pig organs, anti-nonGal antibodies may also be important following adult pig islet transplantation in primates. As adult pig islets are known to express little or no Gal (18-21); anti-nonGal antibodies are likely to be involved in any antibody-mediated rejection process (22-24).

It has sometimes been considered that the baboon is a more difficult preclinical model of xenotransplantation than the macaque monkey. Our data suggest that antibody-mediated xenograft rejection of GT-KO pig organs or cells may be more vigorous in cynomolgus monkeys than in baboons or humans. With regard to the cytotoxicity associated with anti-nonGal antibody binding, the baboon may be closer to the human than the cynomolgus monkey, and therefore may be the preferred experimental recipient of GT-KO pig grafts.

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Chapter 6
Late onset of development of natural anti-nonGal antibodies in infant humans and baboons: Implications for xenotransplantation

P.P.M. Rood, H.-C. Tai, H. Hara, C. Long, M. Ezzelarab,Y.-J. Lin, J. Busch, D.J. van der Windt, D. Ayares, J.N.M. Ijzermans, R.F. Wolf, R. Manji, L. Bailey, D.K.C. Cooper. Transplant International, 2007;20(12):1050-1058

ABSTRACT

If an ABO-incompatible heart is transplanted into an infant before natural antibodies have developed to the specific donor carbohydrate A/B antigen(s), then B cell tolerance to the donor A/B antigen is achieved, and these antibodies never develop. Anti-carbohydrate antibodies play a role in the rejection of wild-type (WT) and α1,3-galactosyltransferase gene-knockout (GT-KO) pig xenografts. We investigated development of these antibodies in infant baboons and humans. Serum samples from infant baboons (n=42) and humans (n=42) were tested by flow cytometry for IgM and IgG binding to peripheral blood mononuclear cells (PBMC) from WT and GT-KO pigs, and for complement-dependent cytotoxicity. The presence of anti-blood group antibodies was tested in baboon serum. In infant baboons and humans, cytotoxic anti-Galα1,3Gal antibodies develop during the first 3 months, and steadily increase with age, whereas cytotoxic anti-nonGal antibodies are either absent or minimal in the majority of cases throughout the first year of life. Anti-blood group antibodies were not detected before 16 weeks of age. Our data suggest GT-KO pig organ/cell transplants could be carried out in early infancy in the absence of preformed cytotoxic anti-nonGalα1,3Gal antibodies.

INTRODUCTION

There is a critical shortage of human organs for transplantation, particularly in infants requiring heart transplants. The Toronto group has utilized hearts from ABO blood group-incompatible donors to overcome the high mortality in infants waiting for a donor heart (1). They have demonstrated that 'natural' anti-A or anti-B antibodies (Abs) take several weeks or months to develop in infants and that, if an *ABO-incompatible* heart transplant is performed before the development of such Abs, then these Abs *never* develop (2). They have demonstrated that B cell tolerance to the specific A and/or B antigen can be achieved in infants who receive immunosuppressive therapy directed only against the T cell response, which is essentially similar to that used for *ABO-compatible* transplant recipients.

The mechanism by which the primate immune system responds to ABO carbohydrate antigens is believed to be similar to that governing the response to carbohydrate antigens expressed by xenogeneic tissues from genetically-distant species, such as swine (3). This opens the possibility that, if a pig organ is transplanted into an infant before the development of anti-pig Abs, then a state of B cell tolerance to pig carbohydrate antigens may result. The Toronto group's work has therefore suggested a method by which the development of natural anti-pig Abs could be prevented. Since an immunosuppressive regimen based on a combination of an anti-CD154 monoclonal Ab and mycophenolate mofetil has been demonstrated to prevent the T cell-dependent elicited Ab response in pig-to-baboon organ

and cell transplant models (4-8), pig organ transplantation could theoretically be carried out in infants in the absence of ***all*** (natural and elicited) Abs directed to pig antigens.

Natural anti-pig Abs develop in humans during the first few months of life, apparently as a response to colonization of the gastrointestinal tract by various microorganisms (9). Natural Abs against wild-type (WT) pigs include both those directed to Galα1,3Gal (Gal) antigens and those directed to other unidentified antigens, referred to as nonGal; these may be carbohydrate or protein antigens. In contrast, GT-KO pigs express only nonGal antigens. The majority of primate anti-pig Abs are directed to Gal epitopes on the WT pig vascular endothelium and certain other tissues (10-13), and which are also present on various bacteria and viruses (9). In nonhuman primates and humans, binding of anti-pig Abs, particularly anti-Gal Abs, to transplanted pig organs results in complement activation and hyperacute rejection.

The problem of anti-Gal Abs has been overcome by the generation of pigs homozygous for α1,3-galactosyltransferase gene-knockout (GT-KO), which renders them incapable of producing the Gal oligosaccharide (14, 15). However, other preformed anti-pig Abs directed to nonGal antigens are present in approximately 50% of adult humans and Old World nonhuman primates (16), and are cytotoxic to pig cells, and may result in a delayed form of Ab-mediated rejection known as acute humoral xenograft rejection (5, 6, 17). A method of preventing the development of natural anti-pig Abs would therefore greatly enhance the prospect of successful pig organ transplantation in primates since it would negate the injury caused by such Abs.

The presence of anti-Gal Abs in newborn baboons was initially investigated several years ago (18-23). Only maternal IgG was present in the tested serum samples from neonates. Although the transplantation of an unmodified WT pig organ into an untreated baboon during the first few weeks of life rapidly led to the development of elicited anti-Gal IgG that caused AHXR, the absence of preformed anti-Gal IgM meant that hyperacute rejection did not occur. These studies therefore suggested that the transplantation of a WT pig organ very early in life, in the presence of no, or only low levels of, anti-pig IgM, is associated with an absence of hyperacute rejection.

However, little is known about the rate of development of anti-pig Abs in the first year of life, particularly in regard to anti-nonGal Abs. We have investigated the development of anti-pig Abs, and specifically anti-nonGal Abs, in both infant baboons and humans, and we address the implications that our findings may have for pig organ transplantation into newborns. We hypothesized that infant humans and baboons would not have significant levels of anti-pig Abs in the first few months of life, and that anti-nonGal Abs may increase at a slower rate during the first year of life. We also hypothesized that the pattern of change between infant

humans and baboons would correlate such that baboons would be considered a suitable surrogate for humans in preclinical studies.

MATERIALS AND METHODS

Sources of Serum

Humans

Serum samples (n=42) from humans (age <1-64 weeks) were from patients being assessed for a variety of reasons, including cardiac surgical procedures (Loma Linda University, Loma Linda, CA, and University of Alberta, Edmonton, Canada). Samples were tested for IgM and IgG binding to GT-KO (n=42) and WT (n=33) pig peripheral blood mononuclear cells (PBMC) by flow cytometry, and 41 samples were tested for cytotoxicity.

The Edmonton Health Research Ethics Board required documentation of informed consent prior to acquisition of infant serum samples, and this was accomplished. Because of the following caveat, the Loma Linda Institutional Review Board deemed that the need for consent from parents or legal guardians was unnecessary. Loma Linda human serum samples, obtained for clinical purposes other than this research, were identified and salvaged (before being discarded) by assigned third-party laboratory technicians. Samples were labeled on the basis of donor age alone, before delivery to the principal investigators. Hence, patient identity and right to know would not be violated or traceable. This represents a well-considered difference of opinion between the two human studies review boards, and is not germane to the present study.

Baboons

Serum samples (n=56) were collected from 42 baboons (age 6-70 weeks) (OUHSC, Oklahoma City, OK). Forty samples were from 26 colony-raised infant baboons, of which 14 were tested a second time (23 weeks after the first sampling). The other 16 samples were from baboons maintained under specific pathogen-free (SPF) conditions. All initial serum samples were tested for IgM and IgG binding to WT and GT-KO pig PBMC by flow cytometry and for complement-dependent cytotoxicity, but the 14 samples retested after 23 weeks were tested only for cytotoxicity.

All animal care procedures were in accordance with the *Principles of Laboratory Animal Care* formulated by the National Society for Medical Research and the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication No. 86-23, revised 1985).

Sources and Isolation of Porcine PBMC

To detect the presence of anti-pig (Gal+nonGal) and anti-nonGal Abs in these sera, PBMC were collected from the blood of a single WT and a single GT-KO (14) pig, to avoid variability between different WT and GT-KO pig cells. Both were of Large White/Landrace/Duroc cross-breed (Revivicor, Blacksburg, VA). (Several studies in our laboratory have demonstrated that there is little variability in IgM/IgG binding and serum cytotoxicity to PBMC from different pigs from the same source.) The PBMC were used as targets in flow cytometry and cytotoxicity assays. The GT-KO pigs differed from the WT pigs only with regard to the absence of Gal epitopes (14). Isolation of PBMC was performed by gravity centrifugation, as previously described (16).

Flow Cytometry

IgM and IgG binding to WT and GT-KO PBMC was determined by flow cytometry, as previously described (16). Briefly, isolated PBMC were resuspended in FACS-buffer (PBS containing 1% bovine serum albumin [Gibco, Grand Island, NY,USA] and 0.1% NaN_3) to a final cell concentration of $12.5x10^6$ cells/mL. One million PBMC were then removed and incubated with 20μL of heat-inactivated primate serum (at 56°C for 30min), diluted in FACS buffer to 20% serum final concentration, for 30min at 4˚C. After incubation, the cells were washed twice in 2mL FACS buffer, vortexed and centrifuged at 1800rpm (700g) for 5min. Cells were then resuspended in 10μL of 10% goat serum in FACS buffer, to prevent non-specific binding, and incubated further with 10μL fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG (γ chain-specific) at 1:50 dilution, or with IgM (μ chain-specific) at 1:200 dilution, (Zymed Laboratories, San Francisco, CA) for 30min in the dark at 4˚C. Antibodies bound to live cells were analyzed using FACScan (Becton Dickinson, Mountain View, CA). Dead cells were excluded from the analysis by forward-scatter and propidium iodide staining (10μL added to each sample prior to FACS analysis). The mean fluorescence intensity (MFI) reading of binding strength correlates with the level of Ab in the serum samples. The high number of samples necessitated the flow cytometry experiments to be carried out in different batches on different days. In order to correct for differences in isotype controls on different days, we used an index. The MFI reading of the experimental sample was divided by that of the (isotype) control sample for that individual batch. The isotype control consisted of the MFI reading of PBMC stained with FITC IgM and IgG only, without the addition of serum. Whenever the index was >1.1, the sample was considered positive. We classified serum samples to have minimal binding (index 1.1-1.5), mild-to-moderate binding (index 1.5-2.5), or strong binding (index >2.5).

^{51}Chromium-release Complement-dependent Cytotoxicity Assay

The complement-dependent cytotoxicity assay measured serum cytotoxicity of PBMC that were labeled with ^{51}chromium (^{51}Cr). The three components (serum + complement + target cell) were mixed in microtiter-plate wells, and lysis of the target cells was detected by measuring the release of ^{51}Cr radioactivity into the cell supernatant.

Briefly, ^{51}Cr-labeled target cells were prepared from PBMC that were washed with PBS and centrifuged for 4min at 4˚C. The supernatant was removed, the pellet was resuspended with 40µL of 10% FBS, and each 1x10^6 PBMC was incubated with ^{51}Cr of 50µCi for 60min at 37˚C. Serum samples were incubated for 30min at 56˚C to inactivate complement. The samples were four-fold serially diluted with MLR medium (RPMI 1640 media [Gibco] +10% FBS) to final concentrations of 25%, 6.0%, 1.5%, and 0.3%. As an interplate control, pooled sensitized human serum was also diluted in this manner and used on all plates. On a round-bottomed 96-well plate, 0.01x10^6 ^{51}Cr-labeled cells (suspended in 80µL MLR medium) were loaded into each well, and incubated with 80µL of diluted serum for 30min at 4°C. After incubation, the cells were further incubated with 40µL of 10% rabbit HLA-ABC serum (Sigma, St. Louis, MO, USA), as a source of complement, for 45min at 37°C.

Minimal killing controls (100% live cells) were achieved by adding 80µL MLR medium or complement to wells containing cells without serum. Maximal killing controls (0% live cells) were achieved by adding detergent (5% (v/v)Triton X-100; Sigma) to wells containing cells without serum.

Following incubation, the cells were centrifuged at 300-400rpm (35g) for 4min. The supernatant containing ^{51}Cr released from lysed cells was harvested using the Macrowell™ Tube Strips collection system (Molecular Devices, Sunnyvale, CA). Cell killing was assessed by reading the ^{51}Cr released from lysed cells using a gamma irradiation counter (INC Biomedical, Costa Mesa, CA).

Cell killing was calculated using the following formula:

$$\% \text{ cytotoxicity} = [(A-C)/(B-C)] \times 100,$$

where A equals the count per minute of sample dead cells (+ serum and complement), B equals the count per minute of maximal dead cells (+ detergent), and C equals the count per minute of minimal dead cells (+ complement only).

Complement-dependent cytotoxicity values of the varying serum concentrations (25%, 6.0%, 1.5%, and 0.3%) were calculated, and a curve was generated for each sample. Results were considered acceptable based on the slope of a curve generated by a graded concentration of human control serum that was added to every plate. Lysis of <10% was considered of doubtful significance.

ABO Blood Typing of Baboons by Buccal Mucosal Smear and Hemagglutination Assay for Anti-A/B Antibodies in Baboon Serum

ABH phenotyping of buccal mucosal smears was accomplished in baboons by immunohistochemical staining, and presence of anti-A/B antibodies in baboon serum was determined by hemagglutination assay, as previously described (24, 25).

Statistical Analysis

Analysis of indirect immunofluorescence intensity was accomplished by Cell Quest software and converted into MFI by WinMDI software. Statistical analyses were performed using tests for paired data: Wilcoxon signed rank test for comparison of medians and McNemar's test for comparison of proportions. Correlation of MFI or cytotoxicity with age was assessed by linear regression analysis. Statistics were calculated using prism-4 software (Graphpad Software, San Diego, CA) and SPSS 14.0 for Windows (SPSS, Chicago, IL) and p-values were compared to the 95% level of significance.

RESULTS

Binding of Infant Human and Baboon Serum IgM and IgG to Pig PBMC by Flow Cytometry

We studied serum samples of infant humans (n=42) and infant baboons (n=56) by flow cytometry for IgM and IgG binding to WT and GT-KO PBMC.

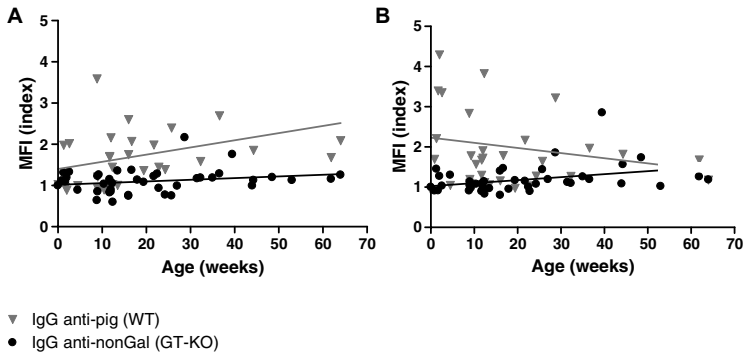

Figure 1:
Binding of infant human (n=42) serum Abs to WT and GT-KO pig PBMC. (MFI index = mean fluorescence intensity of the serum sample divided by the MFI of the isotype control sample).
(A) Distribution of IgM reactivity against WT and GT-KO PBMC. Correlation of MFI index with age of each group is indicated by a line (versus WT, p=0.073, r=0.316; versus GT-KO, p=0.129, r=0.238).
(B) Distribution of IgG reactivity against WT and GT-KO PBMC. Correlation of MFI index with age of each group is indicated by a line (versus WT, p=0.381, r= -0.158; versus GT-KO, p=0.021, r=0.356).

Humans

IgM binding to WT PBMC was seen as early as one week of age, and binding increased with age, though not significantly (p=0.073) (Figure 1a). No or only minimal IgM binding to GT-KO PBMC was seen during the first 30 weeks, and the level of anti-nonGal Abs remained low throughout the first year, with no correlation with age (p=0.129) (Figure 1a). Binding of IgM to WT and GT-KO cells is documented in Table 1. IgM binding to both WT and GT-KO PBMC was lower than in baboons (see below), which was consistent with cytotoxicity findings (see below, Figure 3).

Table 1:
Strength of binding of infant baboon and human serum* IgM and IgG to WT and GT-KO PBMC by flow cytometry

	Human (*n* = 42)				Baboon (*n* = 42)			
	IgM		IgG		IgM		IgG	
	WT	GT-KO	WT	GT-KO	WT	GT-KO	WT	GT-KO
Strength of binding (%)								
No binding	34	48	15	53	5	38	60	98
Minimal	15	48	24	38	28	52	17	0
Mild-to-moderate	30	4	40	7	50	10	21	2
Strong	21	0	21	2	17	0	2	0

*Drawn at various times during the first year of life.

The level of IgG binding to WT PBMC in neonates was high, and decreased during the course of the next few weeks and months, although this fall was not significant (p=0.381) (Figure 1b). (These high IgG levels seen soon after birth were likely due to maternal IgG.) In contrast, the level of IgG binding to GT-KO PBMC was low in neonates (not significantly different from isotype control), but increased with age during the first year of life (p=0.021), although binding remained low.

In neonates (age <4 weeks, n=6), in relation to isotype control, IgG binding to WT PBMC was increased up to approximately four times, while binding to GT-KO PBMC was minimal (Figure 1b) (p=0.046). The maternal anti-pig IgG present in the sera of neonates would therefore appear to be almost entirely anti-Gal Ab.

Baboons

There was no or minimal IgM binding to WT PBMC (as defined by MFI-index<1.5, see Materials and Methods) documented earlier than 10 weeks of age (n=4), after which the

incidence and extent of binding steadily increased with age (p<0.001) (Figure 2a). No or only minimal IgM binding to GT-KO PBMC was seen before 15 weeks, after which there was some increase, but the extent of binding remained low throughout the first year, and there was no correlation with age (p=0.103) (Figure 2a). Binding of IgM to WT and GT-KO cells is documented in Table 1.

There was a slight increase in the level of IgG binding to WT PBMC during the first year, though the increase was not significant (p=0.444) (Figure 2b). The levels of binding to GT-KO PBMC in most cases remained at the level of the isotype control (MFI-index =1), and there was no correlation with age (p=0.740). Binding is documented in Table 1.

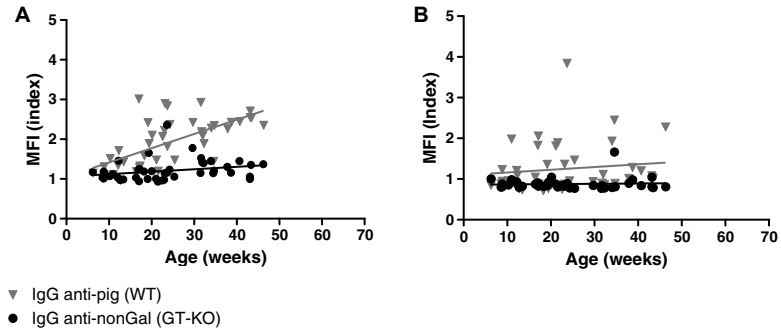

Figure 2:
Binding of infant baboon (n=42) serum Abs to WT and GT-KO pig PBMC. (MFI index = mean fluorescence intensity of the serum sample divided by the MFI of the isotype control sample).
(A) Distribution of IgM reactivity against WT and GT-KO PBMC. Correlation of MFI index with age of each group is indicated by a line (versus WT, p<0.001, r=0.665; versus GT-KO, p=0.103, r=0.255).
(B) Distribution of IgG reactivity against WT and GT-KO PBMC. Correlation of MFI index with age of each group is indicated by a line (versus WT, p=0.444, r=0.121; versus GT-KO, p=0.740, r=0.053).

Sera from colony-raised baboons (n= 26) and SPF baboons (n=16) were investigated separately. No significant difference in IgM or IgG binding to either WT or GT-KO pig PBMC was found using sera from these two groups (data not shown), suggesting that anti-pig Ab formation was not influenced by the SPF environment.

Cytotoxicity of Infant Human and Baboon Sera to Pig PBMC

The same serum samples were tested for complement dependent cytotoxicity against WT and GT-KO PBMC. All samples showed a titration curve with reduction in lysis with increasing

dilution (not shown). In order to demonstrate correlation of cytotoxicity with age, lysis (= % cytotoxicity) at a serum dilution of 25% is shown in Figure 3.

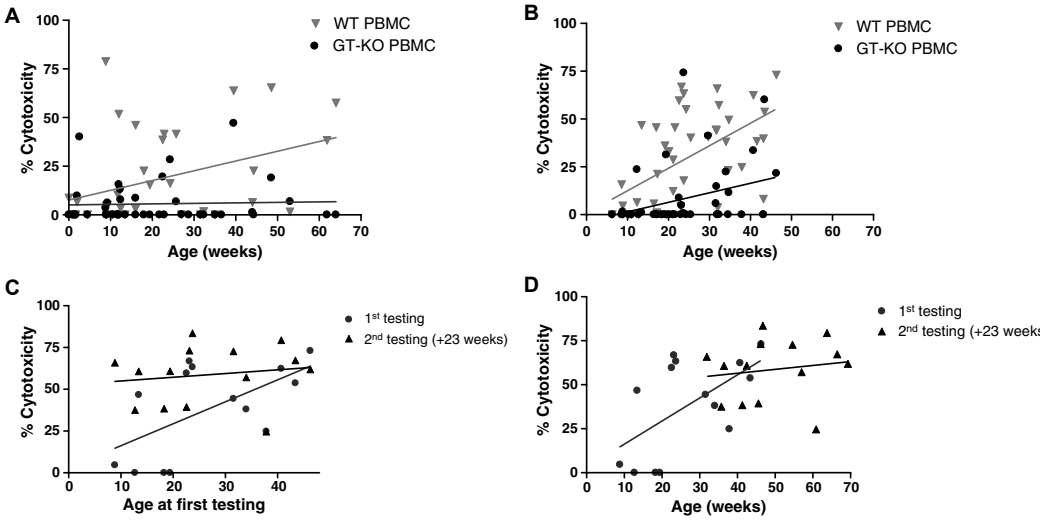

Figure 3:
(A) Ability of human serum samples (n=41) at 25% dilution to cause lysis of PBMC from WT or GT-KO pigs. Correlation of complement-dependent cytotoxicity with age is indicated by lines. Significant correlation with age was observed for lysis of WT PBMC (p=0.023, r=0.377), but not for lysis of GT-KO PBMC (p=0.803, r=0.040).
(B) Ability of baboon serum samples (n=42), tested at 25% dilution, to cause lysis of PBMC from WT or GT-KO pigs. Correlation of complement-dependent cytotoxicity with age is indicated by lines. Significant correlation with age was observed for both lysis of WT PBMC (p<0.001, r=0.522) and GT-KO PBMC (p=0.035, r=0.330).
(C) Ability of baboon serum samples (n=14), tested at 25% dilution on 2 occasions (the second 23 weeks after the first), to cause lysis of PBMC from a WT pig. At the second testing, an increase in cytotoxicity of most baboon anti-pig Abs was documented (n=11).
(D) The same data presented in (C) have been plotted to show the correlation of complement-dependent cytotoxicity with age (indicated by lines). At the initial testing, a significant correlation was observed between lysis and age (circles) (p=0.029, r=0.582). At the second testing (23 weeks later; triangles), an increase in cytotoxicity of baboon anti-pig Abs (compared with the initial testing) was documented, but increasing lysis did not correlate with increasing age (p=0.600, r=0.153).

Humans

During the first year of life, 39% of infant human sera were cytotoxic (>10% lysis) to PBMC from WT pigs, whereas only 17% were cytotoxic to GT-KO PBMC (p=0.002) (Figure 3a). The extent of GT-KO PBMC lysis was significantly less than that of WT PBMC (median percentage of lysis 0% vs 6%, respectively, p=0.001). Lysis of WT PBMC increased significantly with age (p=0.023), but lysis of GT-KO PBMC (i.e., associated with anti-nonGal Abs) did not increase with age (p=0.803). The median lysis of GT-KO PBMC throughout the entire first year was 0% (range 0-40%).

Baboons

During the first year of life, 67% of infant baboon sera were cytotoxic (defined by >10% lysis) to PBMC from WT pigs, whereas only 24% of sera were cytotoxic to GT-KO PBMC (p<0.001). Furthermore, when lysis was detected, the extent of lysis of GT-KO PBMC was generally much less than that of WT PBMC (median percentage of lysis 0% vs. 29%, respectively, p<0.001). Lysis increased significantly with age against both WT PBMC (p<0.001) and GT-KO PBMC (p=0.035) (Figure 3b). Lysis of GT-KO PBMC of >10% was rarely documented in sera from baboons <22 weeks of age (2 of 22 samples). Importantly, 76% of baboons demonstrated no detectable cytotoxicity (i.e. >10% lysis) to GT-KO PBMC.

Sera from 14 infant baboons of different ages were retested for cytotoxicity against WT PBMC 23 weeks after the first blood draw. Cytotoxic anti-pig Abs had increased in these baboons during this interval (median extent lysis increased from 46% to 61%, p=0.012) (Figures 3c and 3d). Infant baboons that were older at the time of the first blood draw showed less increase in cytotoxic anti-pig Abs, suggesting that most anti-pig Abs are formed early in the first year of life.

Presence of Anti-A or B Blood Group Antibodies in Infant Baboons

Blood groups of baboons were determined by staining of buccal swabs. Sera from baboons (n=20) with blood group A or B were then tested for the presence of anti-B or A blood group Abs, respectively. Sera from baboons with blood group AB were not followed up, since they never develop anti-A or B Abs. In the sera tested, we did not detect any anti-A or B Abs before the age of 16 weeks (Figure 4). Most sera of baboons of 17 weeks and older demonstrated detectable levels of Abs, although, in some cases, absence of Abs was seen up to 23 weeks of age. This suggests that anti-blood group Abs do not develop in baboons until the fourth month of life, and are present in most baboons >5 months of age.

All baboons with anti-blood group Abs demonstrated detectable levels of anti-WT pig IgM, but IgM binding to WT pig cells was also observed in sera without anti-A/B Abs. We found a correlation between the presence of anti-A/B Abs and a higher level of IgM binding to WT cells (p=0.029), but no correlation was found between the presence of anti-A/B Abs

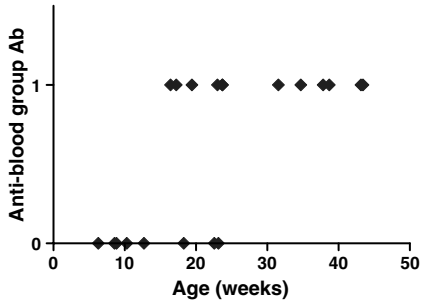

Figure 4:
Presence of anti-A or B blood group Abs in sera of blood group B or A baboons, respectively, of different ages (n=20). (0 = anti-A or B Abs were not detected; 1= anti-A or B Abs were present).

and binding of anti-WT IgG (p=0.357) or of anti-GT-KO IgM (p=0.136) or anti-GT-KO IgG (p=0.715).

DISCUSSION

Natural anti-pig Abs develop during infancy (18-23), a finding confirmed in the present study. However, this is the first investigation of the development of anti-nonGal Abs in infant humans and baboons and the first demonstration of the correlation between age and Ab production. Our data suggest that both baboons and humans probably develop cytotoxic anti-pig IgM early during the first year, the level of which steadily increases with age during the first year, but this IgM is largely directed against Gal targets. Cytotoxic anti-nonGal Abs develop later, and remain at a minimal level in the majority of cases (Figures 1-3). This increase in anti-pig Abs with age during the first year is consistent with that of anti-A/B Abs.

As described in adults (16), the incidence and extent of binding of infant human (Figure 1) and baboon (Figure 2) serum IgM and IgG to PBMC from GT-KO pigs was significantly less when compared to binding to WT PBMC. The associated lysis of WT cells was higher in baboons than in humans (p=0.031) (Figure 3), suggesting that Ab-mediated rejection of a transplanted WT pig organ may be more problematic in the baboon than in the human. The increase in anti-pig cytotoxic Abs was confirmed by sequential measurement of sera from the same baboon (Figure 3). More importantly, the cytotoxicity of infant human anti-nonGal Abs did not increase with age and was absent in a significant number of cases. This allows a 'window of opportunity' during which a GT-KO pig organ could be transplanted into a human infant without the risk of rejection associated with the presence of natural anti-nonGal cytotoxic Abs. Newborns with congenital heart disease, for whom no human donor heart is available, could be possible candidates for receiving a xenotransplant early in life. As occurs in the case of ABO-incompatible heart allotransplantation in human infants (1, 2), B cell

tolerance to carbohydrate antigens may develop when a GT-KO pig heart is transplanted into a primate recipient that has not yet developed anti-nonGal Abs.

However, in contrast to ABO-incompatible allotransplantation, the transplantation of pig hearts might be less successful as cellular components of the innate immune response may be problematic. Furthermore, immune responses to other xenoantigens (against which no natural Abs are present) would need to be suppressed, with the associated risks of immunosuppressive therapy. But it is not definitively known whether immunosuppressive therapy would need to be continued throughout the life of the recipient. Data from Bailey and his colleagues (26, 27) suggest that the immune response to an allograft in neonates and infants is rather less vigorous than in later life, and West et al (1) have drawn attention to the relative 'plasticity' of the neonatal immune system. It would be of great clinical interest to study whether, in this age group, the prevention of a T cell response for a period of time might result in T cell tolerance.

Although our study indicates that anti-nonGal Ab levels in infant sera are frequently absent or low, the presence of a significant level of natural or elicited anti-nonGal Abs is almost certain to result in Ab-mediated rejection (16, 28, 29). The target(s) for these anti-nonGal Abs need to be ascertained (30, 31).

Baboons would generally appear to develop anti-A/B Abs at approximately 4 months of life, which is similar to the time of their development in humans (1). A previous study using adult human and baboon sera showed no statistically significant correlation between the presence of anti-Gal Abs and anti-A/B Abs (32), which is confirmed for IgG Abs by our findings. However, we found a correlation between the presence of anti-A/B Abs and anti-WT IgM in infant baboons, possibly since both levels are known to increase during the first year of life.

There is a discrepancy between the high level of (maternal) anti-pig (Gal) IgG in newborn humans and the relative absence of IgG in newborn baboons. Some or all of this discrepancy is likely to be related to the fact that some human sera were available to us as early as the first week of life, whereas no baboon sera were available until the sixth week. However, the relatively lower levels of IgG seen in the first 3 months in baboons suggest there may be a difference between the two species in this respect, although maternal IgG has been documented in neonatal baboons previously (20).

In summary, our data suggest that GT-KO organ transplants could be carried out in early infancy in human recipients in the complete or relative absence of cytotoxic anti-nonGal Abs. If immunosuppressive therapy were administered to suppress a T cell-mediated elicited Ab response, xenograft rejection resulting from anti-nonGal Abs would be prevented (although other potential problems may still arise (33)). Based on clinical experience of heart allotransplantation in infant humans across the ABO barrier, it is possible that B cell

tolerance to pig carbohydrate antigens might develop. Future studies should focus on testing this *in vivo*, by implanting tissue/organs from GT-KO pigs into infant baboons.

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Chapter 7
Induction of diabetes in cynomolgus monkeys with high-dose streptozotocin: Adverse effects and early responses

P.P.M. Rood, R. Bottino, A.N. Balamarugan, C. Smetanka, M. Ezzelarab, J. Busch, H. Hara, M. Trucco, D.K.C. Cooper. Pancreas, 2006;33(3):287-292

ABSTRACT

Introduction: Streptozotocin (STZ) has been widely used to induce diabetes in nonhuman primates, although it has been found difficult to achieve complete diabetes without serious side effects. We have investigated different types and dosages of STZ, in order to find a way to safely induce complete diabetes in cynomolgus monkeys.

Materials and methods: After adequate hydration, 10 monkeys received STZ. Five monkeys received conventional STZ (Sigma) at a dosage of 1250mg x m^2 ('high-dose') (*n*=4) or 60mg/kg ('low-dose') (*n*=1) (Group I). Five monkeys received Zanosar STZ (Sicor) at 150mg/kg ('high-dose') (*n*=5) (Group II).

Results: 'High-dose' Group I monkeys became completely diabetic (*n*=4), but a protein-losing nephropathy was observed in 3 of the 4 monkeys. The monkey that received 60mg/kg STZ failed to become fully diabetic (C-peptide>1.86ng/mL). Group II (high-dose Zanosar-treated) monkeys became completely diabetic, but with no apparent side effects. A triphasic blood glucose response to STZ was documented in all the high-dose STZ-treated monkeys. Low-dose STZ failed to result in a triphasic response.

Conclusions: (1) High-dose Zanosar STZ induced diabetes safely in cynomolgus monkeys without side effects. (2) A triphasic blood glucose response suggested the complete induction of diabetes.

INTRODUCTION

The growing interest in islet transplantation, supported by results achieved in rodent models, has caused an increase in studies with nonhuman primates (1-5). To assess islet survival, it is preferred to render the nonhuman primates diabetic. Diabetic animals are valuable not only for studying survival after islet transplantation, but also to study several other aspects of diabetes, such as neuropathy (6), the influence of hyperglycemia on cardiac tissue (7), and metabolic and endocrine changes (8).

Spontaneous diabetes has been reported in cynomolgus and rhesus monkeys (9-11), although their availability is limited, and it is necessary to verify the type of diabetes. Pancreatectomy to induce diabetes is not without surgical morbidity, and has the major disadvantage of loss of exocrine pancreatic function (9,12). The tight adherence of the pancreas to the intestine makes pancreatectomy in the monkey a difficult procedure, and there is also the potential for continuing function or regeneration of beta cells if the pancreatectomy has not been total. Although nonhuman primates have been reported to become diabetic after alloxan administration (9), it is generally assumed that alloxan works selectively on rodent beta cells, and is therefore not a potent inducer of diabetes in humans or nonhuman primates (13,14).

Therefore, streptozotocin (STZ), derived from the bacteria *Streptomyces achromogenes*, is a frequently used method for induction of diabetes in nonhuman primates. Besides antimicrobial and anti-tumor activities, STZ is diabetogenic through oxidative stress resulting in selective death of the beta cells (15). Nephrotoxicity and hepatotoxicity are disadvantages of STZ (16-19). There is an ongoing discussion with regard to the optimal dose of STZ in nonhuman primates since there is a well-documented correlation between the dose of STZ and the development of renal and hepatic morbidity.

We here report on our experience with different doses and different types of STZ, in an effort to find a safe and consistent way to induce diabetes in nonhuman primates. We also report on the early metabolic responses (e.g., blood glucose levels) after STZ administration, which may indicate whether diabetes has been induced successfully and completely.

METHODS

Animals

Ten male cynomolgus monkeys (*Macaca fascicularis)*, weighing 3.2 kg ± 0.7kg (range 2.4kg to 4.7kg), were fitted with tethers carrying indwelling intravascular and/or gastric catheters. Tethers provide an advantage with regard to the ease with which blood samples can be obtained and fluids and drugs administered, without the need for restraining or sedating the primate (20). All animal care procedures were in accordance with the *Principles of Laboratory Animal Care* formulated by the National Society for Medical Research and the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication No. 86-23, revised 1985).

Table 1:
Dose, type, infusion time of streptozotocin (STZ), and post-STZ C-peptide values*

Group	Dose of STZ (type)	Infusion Time (min)	C-Peptide Level (ng/mL)
Group 1			
n = 4	1250 mg/m2 (Sigma)†	1–5	<0.5
n = 1	60 mg/kg (Sigma)	1	1.86
			2.93 (after challenge with glucose)
Group 2			
n = 5	150 mg/kg (Zanosar)	1	<0.5

*C-peptide levels measured by chemiluminescence (detection level is 0.5 g/mL).
†≃105 mg/kg.

Induction of diabetes

Monkeys were fasting overnight and were pre-hydrated with 10mL/kg/h 0.9%NaCl iv for 8-10h before STZ administration, to reduce nephrotoxic side effects. After STZ administration, the rate of fluid infusion was increased to 15mL/kg/h for 2h. Five monkeys (Group I) received STZ that was purchased from Sigma Chemical Company (St Louis, MO, USA), four of them at a dose of 1250mg/m^2 body surface area (equals ~105mg/kg, defined as 'high dose'), and one at 60mg/kg (defined as 'low-dose')(Table 1). The other five monkeys (Group II) were given Zanosar STZ (Sicor Pharmaceuticals, Irvine, CA, USA) at 150mg/kg (defined as 'high-dose') (Table 1). In all cases, STZ was administered over 1 to 5min (Table 1).

In the 48h after STZ induction, frequent blood samples were drawn for metabolic testing (including glucose, potassium, HCO_3, pH, pCO_2, acid-base balance) using an i-STAT device for 'bed-side' metabolic blood screening (I-STAT Corporation, East Windsor, NJ, USA). Dextrose was infused continuously, the rate and concentration depending on the blood glucose level. If a metabolic acidosis developed, a continuous infusion of sodium bicarbonate was initiated and continued at a rate to restore the blood acid-base balance (range 0.15-0.9 mEq/hr). Baseline and post-STZ liver and kidney function tests were carried out.

After the successful induction of diabetes, blood glucose levels were checked at least twice daily, and hyperglycemia was controlled by the continuous iv infusion of human recombinant insulin (Humulin R, Eli Lilly, Indianapolis, IN, USA).

Confirmation of induction of diabetes

Diabetes was confirmed by the persistence of fasting hyperglycemia and by the measurement of fasting, random, and glucose-stimulated primate C-peptide. Efforts to stimulate surviving islets were by iv glucose tolerance test (ivGTT), as described by Koulmanda et al. (16), and by the arginine stimulation test, as described by Wijkstrom et al. (2) Serum C-peptide levels were measured by a chemiluminescence method (Bayer Centaur, Tarrytown, NY, USA) and radioimmunoassay (Linco Research, St. Charles, MO, USA) using human antibodies with 100% cross-reactivity with monkey C-peptide. (Since the molecular structures of human and monkey C-peptide are identical). A fasting blood glucose level of >200mg/dL in combination with a stimulated C-peptide level of <0.5mg/mL was considered indicative of diabetes.

Histological examination

After euthanasia of the monkey, biopsies of the pancreas, kidney, liver, and heart were taken and fixed in formalin, embedded in paraffin, and sectioned. Pancreatic tissue was investigated by immunohistochemistry for the presence of immuno-reactive pro-insulin antibodies (Scytek Laboratories, Logan, UT, USA) using standard procedures. Kidney, liver, and heart tissue was stained with hematoxylin and eosin.

Data analysis

Mean values and standard error of the mean (SEM) were calculated using Prism-4 software (Graphpad Software, San Diego, CA, USA).

RESULTS

Pre-STZ data

Pre-STZ C-peptide random values were 2.22-4.24ng/mL measured by chemiluminescence. IvGTT showed normal blood glucose curves (Figure 1) and stimulated C-peptide values up to 7.34ng/mL at 15 min. Arginine stimulation tests showed an increase of C-peptide production to 6.17ng/mL after 2 min. Baseline metabolic blood and urine screening showed normal kidney and liver functions.

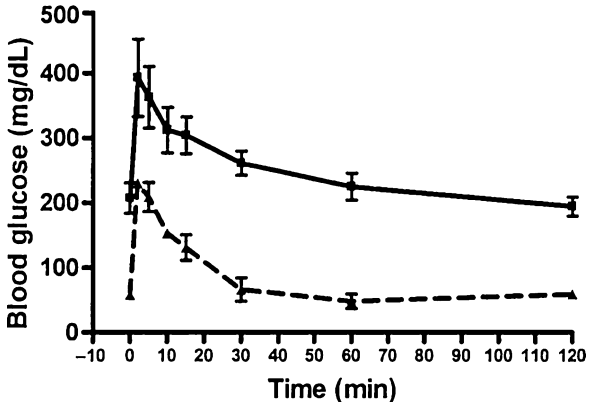


Figure 1:
ivGTT in nondiabetic (n=6) (dashed line) and high-dose STZ-diabetic (n=4) monkeys (solid line).

Clinical response to STZ

All 10 monkeys became consistently hyperglycemic (i.e. blood glucose >200mg/dL) at a mean of 30.3h (range 26.5-34.25h) after STZ administration.

Group I

After high-dose Sigma STZ at 1250mg/m^2, C-peptide measurement demonstrated that all four Group I monkeys became completely diabetic. C-peptide values fell below the 0.5ng/mL detection level, which was confirmed by radioimmunoassay, both when fasting and after iv

glucose challenge on ivGTT testing. IvGTT blood glucose curves confirmed diabetes (Figure 1).

Although consistent hyperglycemia was observed, the Group I monkey that received 60mg/kg Sigma STZ failed to lose complete beta cell function; the fasting post-STZ C-peptide value was 1.86ng/mL, and the glucose-stimulated value was 2.93ng/mL. This monkey showed no side effects post-STZ.

Side-effects were observed in the four monkeys that received high-dose Sigma STZ. These included vomiting within one hour after STZ administration (n=4), and a protein-losing nephropathy was observed (n=3), particularly after immunosuppressive drug therapy was initiated. Serum protein levels came down to an average of 4.3g/dL (range 3.6-5.1), necessitating iv human albumin infusion. Albumin levels decreased proportionately (average 1.7g/dL, range 1.2-2.3). Furthermore, severe proteinuria was documented using urine test strips (Baxter, Deerfield, IL, USA). Serum creatinine levels were stable and within the normal range. Liver transaminases, although not measured in the acute phase, did not show elevation above the normal range post-STZ.

Group II

Group II monkeys became consistently diabetic after 150mg/kg Zanosar STZ administration. Fasting blood glucose was >200mg/dL. Fasting and random C-peptide levels fell below the detection level of 0.5ng/mL by chemiluminescence, and below the 0.1ng/mL detection range by radioimmunoassay. Stimulation with ivGTT and/or arginine did not result in an increase in serum C-peptide, and ivGTT blood glucose curves confirmed diabetes (Figure 1).

Apart from vomiting shortly after STZ administration (n=3), no side effects were observed. Urinalysis did not show proteinuria, and serum protein and albumin levels were stable, indicating the absence of a protein losing nephropathy.

Triphasic blood glucose response

The presence of indwelling intravascular lines enabled us to draw frequent blood samples and monitor the metabolic changes that followed STZ administration. All monkeys that were treated with high-dose STZ (1250mg/m^2 Sigma STZ or 150mg/kg Zanosar STZ) showed a triphasic blood glucose response to STZ (Figures 2 and 3). Initially, over approximately 8h, there was a marked increase to a mean of 330mg/dL followed by a steady reduction to 56mg/dL at 17h (at this point the dextrose infusion was increased). Metabolic acidosis occurred mainly during the second phase, when the blood glucose fell to low levels after the peak at 8h post-STZ administration, necessitating iv bicarbonate infusion. This was followed by a second, and sustained, increase in glucose to a mean of 310mg/dL at 30h (necessitating iv insulin administration).

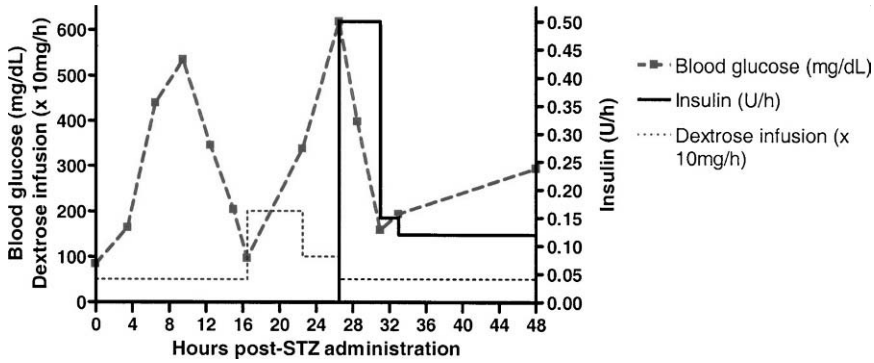


Figure 2:
Representative triphasic blood glucose response (dashed line) after (Zanosar) STZ administration in a representative cynomolgus monkey. Dextrose was administered continuously (indicated in mg/h, dotted line), and insulin treatment was started after 26.5h (solid line).

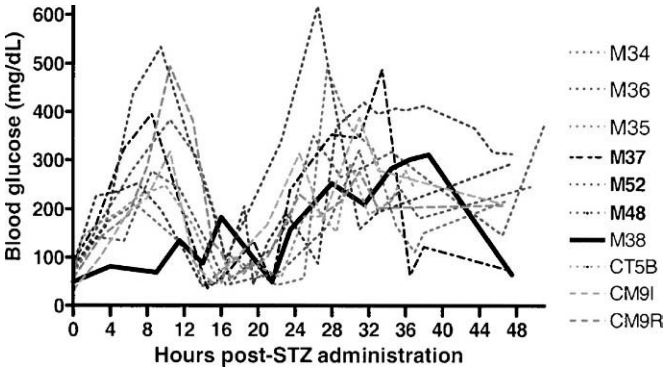
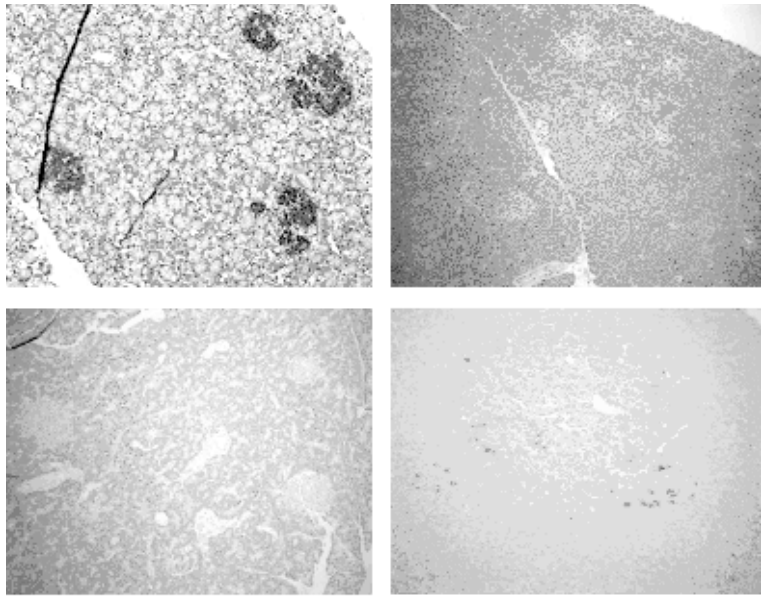


Figure 3:
Blood glucose responses (dashed lines) of individual monkeys (n=9) during and after the successful induction of diabetes after receiving high-dose STZ. A triphasic response can be seen in every case. The aberrant response in one monkey (M38) that was not completely rendered diabetic (after 60mg/kg STZ) is shown in a solid black line.



Chapter 7, Figure 4:
Insulin-positive cells in pancreatic tissue of normal monkey **(A)** and after 150mg/kg Zanosar STZ **(B)**, 1250mg/m2 (~105mg/kg) Sigma STZ **(C)**, and 60mg/kg Sigma STZ **(D)**. No insulin-positive cells are seen after high-dose STZ **(B, C)**; there are still insulin-positive cells present after low-dose STZ **(D)**.

Histology

Pancreatic tissue sections from monkeys that were treated with high-dose STZ (1250mg/m^2 or 150mg/kg) resulted in low or absent insulin staining (Figure 4). Hematoxylin and eosin staining of kidney, liver, and heart sections, taken at the time of euthanasia (*not* in the acute phase following STZ administration) did not show significant lesions in any monkey.

DISCUSSION

Diabetes can be consistently induced after high-dose STZ administration. Both 1250mg/m^2 (~105mg/kg) Sigma STZ as well as 150mg/kg Zanosar STZ resulted in the cessation of C-peptide production (i.e., to a level below the detection level of the respective assays).

There is an ongoing discussion with regard to the optimal dose of STZ in nonhuman primates. Pitkin and Reynolds (21) and Litwak et al. (22) showed that a single dose of 30mg/kg is insufficient to induce complete diabetes. Larger doses of 100-150mg/kg were found sufficient (16,18,23), but were associated with more side effects and complications (16-19,23). In contrast, Koulmanda et al. (16) reported that 55mg/kg was sufficient to consistently induce diabetes in cynomolgus monkeys, but others have been less successful with this dosage (17,21,24,25), with C-peptide production still detectable (in the range of 0.6 to 0.9ng/mL). Wijkstrom et al. (23) suggested the dose should be based on body surface area (1250mg/m^2), rather than body weight.

The islets of younger nonhuman primates may be more resistant to destruction, and this may account for the varying STZ dosages required to induce diabetes (16,24). Furthermore, since STZ has a half-life of only 10 min (16), it seems reasonable to anticipate that higher dosages are necessary if it is not administered rapidly as a bolus.

The correlation between the dose of STZ and the development of renal and hepatic damage is well-documented. This is demonstrated by an increase in blood urea nitrogen, serum creatinine, and/or hepatic enzymes, or by histological injury (16-19). It is therefore of importance to find the optimum dose to successfully induce diabetes with minimal or no risk of side-effects.

To our knowledge, the present study is the first to describe the safe induction of diabetes with high-dose Zanosar STZ, resulting in undetectable (fasting and stimulated) primate C-peptide. Zanosar STZ is used in patients with metastatic islet cell carcinoma, and may have fewer side effects, perhaps because of greater purity and less variability (S. Deng, personal communication). At 150mg/kg, we were able to successfully establish diabetes in nonhuman primates without residual beta cell function, and without associated morbidity. In contrast, high-dose Sigma STZ was associated with adverse effects, although the role of additional immunosuppressive therapy in the development of this damage remains uncertain.

Figures 5A and B present extrapolated data that show the relation between dosages calculated on the basis of body surface area and body weight, for monkeys of different body weights. These indicate that an STZ dosage of 150mg/kg, when converted to body surface area, is much higher than a dose based on body surface area of 1250mg/m^2, particularly in heavier (older) monkeys (Figure 5A). However, in our experience with monkeys up to 4.7kg, we found that 150mg/kg Zanosar STZ safely induced diabetes. Similarly, if the STZ dosage is based on a body surface area of 1250mg/m^2, heavier (older) monkeys would be receiving a dose much lower than 150mg/kg (Figure 5B).

In the present study, a triphasic response in blood glucose was observed during the first 36h after STZ administration. Such a response has been described in rodents (26,27) and

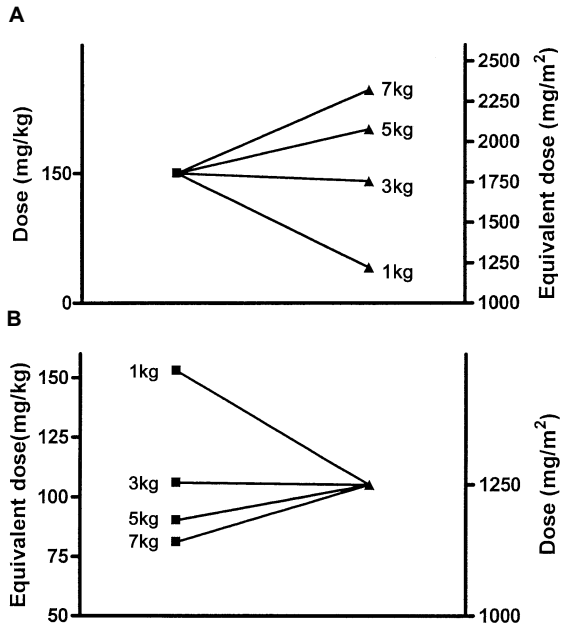


Figure 5:
(A) Extrapolated data showing equivalent doses (calculated per m2 body surface area) of STZ administration at 150 mg/kg body weight for nonhuman primates weighing 7kg, 5kg, 3kg, and 1kg. **(B)** Extrapolated data showing equivalent doses (in mg/kg body weight) of STZ administration at 1250 mg/m2 body surface area calculated for nonhuman primates weighing 7kg, 5kg, 3kg, and 1kg.

monkeys (24,28). Interpretation of the triphasic response is that there is an initial decrease of insulin release as a result of inflammation and an inability of the beta cells to respond to glucose due to impaired glucose oxidation in the beta cell (15,27); this explains the initial rise in blood glucose (phase I). Further destruction and disruption of the beta cells results in a massive release of insulin, leading to a fall in blood glucose level (phase II). Finally, there is a critical loss of beta cells (phase III), resulting in the diabetic state (15,26). We documented a peak blood glucose after 8h (compared to 2-7h in the rat), followed by a steady fall until 17h (8-12h in the rat) and a subsequent rise with persistent hyperglycemia (>200mg/dL) after 30.3h (24h in the rat) (26, 27). Since the monkey that did not become successfully diabetic lacked this triphasic response (Figure 3), we suggest this response indicates the complete and successful induction of diabetes.

In summary, in Zanosar STZ we found a safe agent to completely induce diabetes in monkeys. This may have important implications for those who are establishing a model of diabetes in nonhuman primates. We have shown that a triphasic blood glucose response post-STZ indicates complete and successful induction of diabetes.

ACKNOWLEDGEMENTS

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Chapter 8
Reduction of early graft loss after intraportal porcine islet transplantation in monkeys

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ABSTRACT

Background: Pig islets constitute a possible resolution to the shortage of human islets for transplantation (Tx). After intraportal infusion of porcine islets in primates, many islets are lost through what has been termed the instant blood-mediated inflammatory reaction (IBMIR). We report on our experience with IBMIR.

Methods: Ten monkeys underwent intraportal porcine islet Tx. Immunosuppressive therapy was with conventional agents (n=3) or based on costimulation blockade (n=7). Treatment specific for IBMIR was administered in 8 monkeys, 2 additional monkeys received no such therapy (Group 1). Cobra venom factor (CVF) completely inhibited complement activity in 4 (Group 2), and dextran sulfate (DS) provided anticoagulation, in 4 (Group 3). Islet graft function was monitored by following blood glucose, insulin requirement, and porcine C-peptide values.

Results: In monkeys that received neither CVF nor DS (Group 1), there was rapid destruction of islets, indicated by severe hypoglycemia and the need for dextrose infusion; C-peptide levels were initially low and further reduction occurred within the first 5 days. In both Groups 2 and 3, there was significantly less destruction of islets, and some reversal of diabetes. However, when 40,000IEQ/kg were infused, normoglycemia was lost within 5 days, but when 80,000IEQ/kg were infused in one case, normoglycemia was more persistent. We observed that, even when C-peptide levels were in the normal range for healthy non-diabetic pigs, these were not sufficient to maintain normoglycemia in the monkeys.

Conclusions: Although pre-Tx complement depletion or anticoagulation reduces porcine islet xenograft loss significantly, neither alone is sufficient to prevent IBMIR.

INTRODUCTION

Recent experience has demonstrated the feasibility of treating type 1 diabetic patients by islet allotransplantation (Tx) (1-3), but the shortage of human donor organs is a major limitation. Pig islets form a possible alternative.

Islets are transplanted most commonly intraportally, due to the potential to produce insulin after exposure of the islets to portal blood glucose. The disadvantage of this approach is that direct exposure of the pig islets to the blood can result in their early destruction (4, 5), a phenomenon termed the 'instant blood-mediated inflammatory reaction' (IBMIR). Islets injected intraportally, whether they be allo (6,7) or xeno (5) islets, are subject to this response, which is manifest by activation and consumption of platelets, activation of neutrophils and monocytes, and activation of the coagulation and complement systems (5, 8). Islet Tx also results in activation of both humoral and cellular responses and, indeed, the innate immune system may be involved in the IBMIR.

Medium-term survival of porcine islets in nonhuman primates has recently been reported in the apparent absence of specific treatment for IBMIR (9, 10). However, there is still a need to study and prevent IBMIR, since it would be ideal to obtain normoglycemia with the smallest possible number of islets and with minimal immunosuppression.

The prior administration of soluble complement receptor-1 reduces this rapid islet destruction both *in vitro* and *in vivo* (5), supporting the hypothesis that complement activation plays a major role in IBMIR. Low molecular weight dextran sulfate (DS) has recently been demonstrated to block all aspects of IBMIR (11-13), but its effect on islet destruction after Tx in a nonhuman primate model has not been previously reported.

We here report on our experience with IBMIR after the Tx of adult pig islets intraportally into cynomolgus monkeys. We have focused primarily on observations made during the first 5 days after islet Tx. We observed early graft loss, and assessed the protective effect of the anti-complement agent, cobra venom factor (CVF) combined with heparin, and of DS, which is reported to have both complement-inhibitory and anticoagulant effects (12, 13).

METHODS

Animals

Eight pigs acted as sources of islets. Six outbred large white (wild-type, WT) pigs (Wally Whippo, Enon Valley, PA) were retired breeder sows, and 2 were α1,3-galactosyltransferase gene-knockout (GT-KO) female pigs (14) (Revivicor, Blacksburg, VA) that had never been pregnant (Table 1). All pigs weighed >150kg. Since isolation of WT pig islets resulted in absence of Gal1,3Gal (Gal) expression, we do not believe the source of the islets played any role in the outcome of the experiments in this study. However, we and others have documented that the pancreas from retired breeders provides a greater number of islets with improved insulin production (15, 16).

Ten male cynomolgus monkeys (*Macaca fascicularis)*, weighing 3.3kg±0.7kg (range 2.4-4.2kg) were recipients of the islets. They were fitted with tethers carrying indwelling intravascular and/or gastric catheters, which provide an advantage with regard to the ease with which blood samples can be obtained and fluids and drugs administered, without the need for restraint or sedation of the primate (17).

All animal care procedures were in accordance with the *Principles of Laboratory Animal Care* formulated by the National Society for Medical Research and the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication No. 86-23, revised 1985).

Table 1:
Number and type of islets transplanted, immunosuppressive protocols, and treatment to prevent early graft loss

Monkey no.	IEQ/kg	Immunosuppressive protocol	Treatment specific for IBMIR
M3804[a]	27,000 WT retired breeder	ATG, Tac	Heparin 70 U/kg
M3604	40,000 WT retired breeder	ATG, Tac, Rapa, rituximab	Heparin 70 U/kg
M3404	40,000 WT retired breeder	ATG, Tac, Rapa	CVF, heparin 70 U/kg
M4804[c]	40,000 WT retired breeder	ATG, anti-CD154 mAb, MMF	CVF, heparin 105 U/kg, prostacyclin, aspirin
M5204	40,000 WT retired breeder	ATG, anti-CD154 mAb, MMF	CVF, heparin 105 U/kg, prostacyclin, aspirin
CM9I	40,000 GT-KO non breeder	ATG, anti-CD154 mAb, MMF	CVF, heparin 140 U/kg, prostacyclin, aspirin
CM9R	40,000 GT-KO non breeder	ATG, anti-CD154 mAb, MMF	DS, prostacyclin, aspirin
M8305	40,000 GT-KO non breeder	ATG, anti-CD154 mAb, MMF	DS, prostacyclin, aspirin
M8405[c]	80,000 WT retired breeder	ATG, anti-CD154 mAb, MMF	DS, prostacyclin, aspirin
M8605[b,c]	40,000 WT retired breeder	ATG, anti-CD154 mAb, MMF	DS, prostacyclin, aspirin

[a] Monkey incompletely diabetic.
[b] Monkey nondiabetic.
[c] Received additional islets (15,000–20,000 IEQ/kg) under the renal capsule; M4804 also received additional islets (40,000 IEQ/kg) in an omental pouch.
IBMIR, instant blood-mediated inflammatory reaction; ATG, antithymocyte globulin; CVF, cobra venom factor; DS, dextran sulfate; GT-KO, α1,3-galactosyltransferase gene-knockout; MMF, mycophenolate mofetil; Rapa, rapamycin; Tac, tacrolimus; WT, wild-type.

Surgical procedures

Under general anesthesia, intravascular lines were inserted in the common carotid artery and the jugular veins. In some animals an intragastric line was placed. The animals were tethered and remained with indwelling catheters throughout the period of induction of diabetes and intraportal islet Tx, which was performed under direct vision through a laparotomy under general anesthesia. The numbers of islets transplanted are summarized in Table 1. In 3 monkeys, additional islets were placed under the kidney capsule, and in one of these monkeys they were also placed in an omental pouch (Table 1).

Induction of diabetes

Diabetes was induced with streptozotocin in nine monkeys. Eight monkeys received high-dose streptozotocin (105-150mg/kg iv) from Sigma (n=4) or Sicor (Zanosar; n=4), as previously described (18). These monkeys became completely diabetic with undetectable endogenous C-peptide values. One monkey received a low dose of streptozotocin (60mg/kg) and became hyperglycemic, but maintained the capacity to produce insulin endogenously (18) (Table 1). One monkey received no streptozotocin.

Islet isolation and preparation

Following pancreatectomy in the anesthetized donor pig, the isolation of pig islets was carried out according to a modification of the method described for human islets and further optimized for pigs (19, 20). We used Liberase-PI (Roche, Indianapolis, IN) from multiple lots, and a large-size (700mL) Ricordi digestion chamber maintained at <34ºC, with very gentle mechanical shaking. Enzymatic digestion was discontinued by diluting the enzyme solution

with cold RPMI-1640 (Life Technologies, Gaithersburg, MD), supplemented with 10% heat-inactivated fetal calf serum (Life Technologies) (21, 22). Separation of islet-enriched fractions was performed using discontinuous gradients (densities of 1.132, 1.108, 1.096, and 1.037) in a COBE 2991 Cell Separator (Gambro BCT, Lakewood, CO) (23, 24). Purity was evaluated after dithizone staining of islet samples, and expressed as percent of islets/whole tissue (23). Islets were cultured overnight in CMRL-1066 medium (Mediatech, Herndon, VA), supplemented with 10% heat-inactivated porcine serum (Life Technologies), 10units/mL penicillin, 100mg/mL streptomycin (Invitrogen, Carlsbad, CA), and 10mM nicotinamide (Sigma-Aldrich, St. Louis, MO), at 37ºC, in an atmosphere of 95% air and 5% CO_2.

Islet assessment in vitro and in vivo

Functional properties of the islets, including dynamic perifusion and viability, were determined *in vitro* prior to Tx, as described previously (25). For glucose challenge assay, islets were subjected to low glucose (2.8mM), high glucose (16.7mM), and high glucose plus theophylline (10mM) challenges. *In vivo* Tx studies were conducted in mice, as previously described (26).

Immunofluorescence studies

Sections of isolated porcine islets were fixed in 2% paraformaldehyde and frozen. Using standard procedures, islets were stained with fluorescein-labeled *Griffonia simplicifolia*-1 isolectin B4 (1:100; Vector laboratories, Burlingame, CA) for Gal expression. For insulin immunostaining, sections were incubated with rabbit anti-insulin IgG (1;250; Santa Cruz Biotech, Santa Cruz, CA) followed by Cy3-conjugated goat anti-rabbit secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA). Stained islets were examined under an Olympus Fluoview BX61 confocal microscope (Olympus, Melville, NY).

Islet transplantation

Prior to transplantation, pig islets were counted, and viability was assessed by double fluorescent calcein-AM and propridium iodide stain (27). Islet cell viability was >75% in all preparations, and purity was >80% islets/whole tissue. Islets were transplanted intraportally in plain CMRL-1066, either in heparin (70-140U/kg; Baxter, Deerfield, IL) (n=6) or in DS (MW ~5000d; 1.5-4.5mg/kg; Fluka, Buchs, Switzerland) (n=4) (Table1).

Immunosuppressive and other drug therapy

The immunosuppressive agents that were administered are summarized in Table 1. Anti-thymocyte globulin (ATG) (Thymoglobulin®, generously provided by Genzyme, Cambridge, MA) was given iv over 7-10h on days -3 (n=10) and -1 (n=9) at 6-10mg/kg and 1-10mg/kg, respectively, to reduce the peripheral blood $CD3^+$ T cell count to approximately 500cells/μL (determined by flow cytometry). To prevent side effects from ATG administration, the animals were pretreated with diphenhydramine (5mg/kg iv), metoclopramide (0.5mg/kg iv), and methylprednisolone (5mg/kg iv).

Tacrolimus (Astellas, Deerfield, IL) therapy was initiated on day -7 at 1.5mg/kg orally through an intragastric tube x2 daily, aiming for a trough level of 4-6ng/mL. Rapamycin (A.G. Scientific, San Diego, CA) was started on day -7 at 0.5-1.5mg/kg daily orally through an intragastric tube, aiming for a trough level of 9-15ng/mL. One monkey (M3604) received Rituximab (375mg/m^2; Genentech, San Francisco, CA) on day 0 after islet Tx, and again on day 5 after Tx.

A human anti-human CD154 monoclonal antibody (ABI 793, generously provided by Novartis Pharma, Basel, Switzerland) was administered at 25mg/kg at days -1, 0, 4, 7, 10, 14, and then every 5 days to achieve a whole blood trough level of >400ng/mL (determined by ELISA) (28). Mycophenolate mofetil (MMF; Roche, Nutley, NJ) was given by continuous iv administration at 75-150mg/kg/day iv, beginning on day -4, aiming for a constant level of 3-5µg/mL. One monkey (M8305) received MMF orally x2 daily.

Heparin was administered with the islets (70-140U/kg), but not when DS was given. CVF (Advanced Research Technologies, San Diego, CA) was given to deplete complement from day -1 until day 4, and consistently resulted in CH50 values of 0-5% from day 0 before islet Tx. DS was administered as a bolus (3-10mg/kg iv) 15min before islet Tx; in addition, 1.25-4.5mg/kg was given intraportally with the islets, and thereafter DS was given as a continuous iv infusion (at 0.5-2mg/kg/h) for 6 hours - 5 days. (In the initial protocol, DS was to be administered for 5 days, but after some minor bleeding complications, the period was greatly reduced [see below]). Activated clotting time (ACT) and/or activated partial thromboplastin time (aPTT) values were measured regularly. The effects of heparin and DS were monitored by measurement of the ACT or aPTT, and the doses modified to prevent excessive anticoagulation (i.e., to maintain the ACT <225 and the aPTT <90sec). In 7 monkeys, prostacyclin (GlaxoSmithKline, Research Triangle Park, NC) was administered by continuous iv infusion at 80ng/kg/min, beginning 30min before islet Tx and continued for at least 7 days for its suppressive effect on endothelial cell activation, and aspirin (81mg x2 daily) was started on day -4 for its anti-inflammatory effect and to reduce platelet aggregation (Table 1).

Supportive therapy

After insertion of the intravascular lines, famotidine (0.25mg/kg) or cimetidine (10mg/kg) was administered iv x2 daily. When immunosuppressive therapy was initiated, all monkeys received daily ganciclovir (5mg/kg iv daily) and levofloxacin (10mg/kg iv daily) or cefazolin (10mg/kg iv x2 daily) as prophylaxis against infection.

Monitoring

Complete blood count, electrolyte levels, and tests for acid-base balance and selected renal and hepatic functions, were carried out frequently. Blood glucose values were measured at least x2 daily before islet Tx, and more frequently after Tx. Before islet Tx, regular

insulin (Humulin® R; Eli Lilly, Indianapolis, IN) ± 5% dextrose was administered, aiming at maintaining the blood glucose between 100-200mg/dL to prevent severe hyper- or hypoglycemia. After islet Tx, the period of normoglycemia in the absence of insulin therapy was measured. Complement activity was determined with the Diamedix EZ Complement CH50 test (IVAX Diagnostics, Miami, FL), according to the manufacturer's protocol**.** Porcine C-peptide levels were measured by radioimmunoassay (Linco Research, St. Charles, MO) at approximately 8h and 20h after Tx, and subsequently at intervals of 1-4 days, according to the manufacturer's instructions.

Study groups

Based on whether the monkey received any specific therapy aimed at reducing the IBMIR, the islet graft recipients have been divided into 3 groups (Table 1). Group 1 (n=2) received no IBMIR-directed therapy except heparin with the pig islets. Group 2 (n=4) received heparin with the islets and a 5-day course of CVF. Group 3 (n=4) received DS immediately before the islet Tx (as an iv bolus), with the islets (as a second bolus), and subsequently iv for between 6h and 5 days, but no heparin or CVF. Three of the four monkeys in Group 2 and all four in Group 3 received identical immunosuppressive and supportive therapy, and therefore are comparable in this respect. In view of the fact that we found that DS had little effect on complement activity, Group 2 represented islet Tx in a recipient totally depleted of complement (but not significantly anticoagulated), and Group 3 represented islet Tx in an anticoagulated recipient (in which complement activity had been only minimally reduced).

Statistical analysis

Statistical analysis of data was performed using Student's t test (SPSS for Windows version 13.0 [SPSS Inc., Chicago, IL]).

RESULTS

Quality of porcine islets

Each islet preparation utilized for Tx was assessed for functional properties *in vitro* as well as *in vivo*. Islets were subjected to dynamic perifusion using glucose (2.8 vs. 16.7mM) with and without theophylline (10mM) as stimuli for insulin secretion. The ratio between stimulated and basal insulin release (stimulation index) was 2.6±0.8 after high glucose exposure, and 5.6±1.8 following high glucose and theophylline. Islet viability was assessed using a dual immunofluorescence staining method (calcein-AM and propidium iodide) and was >75% in all preparations. Additionally, using an islet marginal mass composed of 200 to 350 islets (differences in number to account for variation in islet size) from 6 donor islet preparations (including one GT-KO), islets were transplanted under the kidney capsule of 17 STZ-diabetic immunodeficient (NOD-scid and Rag) mice. Fifteen animals normalized, 1 did not, and 1 died shortly after surgery. No statistical difference between WT and GT-KO

islets was noted when comparing *in vitro* or *in vivo* functional performance. Morphological inconsistency between pig islet preparations, with regard to degree of islet integrity, were observed, but were consistent with the difference between truly retired-breeder and merely adult islet donors.

Early islet graft loss

Early islet destruction after Tx results in insulin release from the islets, which in turn leads to hypoglycemia. As an estimation of the extent of islet graft loss, in the completely diabetic monkeys (n=8) we calculated the amount of dextrose administered iv to prevent hypoglycemia (blood glucose <80mg/dL) within the first 2h and also within the first 8h after Tx. In order to correct for differences in body weight and differences in the number of islets transplanted (islet equivalents; IEQ), the amount of dextrose infused was calculated per kg body weight of the recipient monkey, and per 10,000IEQ transplanted. As soon as insulin therapy was required to reverse hyperglycemia, 5% dextrose was infused at a standard rate of 5mL/h; therefore, at this point, we stopped measuring the amount of dextrose infused.

We observed an important difference between the amount of dextrose administered to monkeys that did not receive any specific treatment to prevent IBMIR, and thus early loss of islets (Group 1), and those that received either CVF (Group 2) or DS (Group 3). In the absence of any agent intended to deplete complement and with only one bolus of 70U/kg heparin to provide anticoagulation (Group 1), the amount of dextrose that was infused to correct hypoglycemia during the first 2h (30mg/kg/10,000IEQ transplanted) and 8h (55mg/kg/10,000IEQ) was considerable. The dextrose requirement was significantly less in both Group 2 (CVF+heparin; 4.7 at 2h [$p<0.01$] and 21.7 at 8h [$p<0.05$]) and Group 3 (DS; 2.7 [$p<0.01$] and 19.7 [$p<0.05$] at 2h and 8h, respectively). The dextrose requirements in Groups 2 and 3 were not significantly different ($p=0.47$ at 2h and $p=0.89$ at 8h).

This indicated that CVF or DS protected the porcine islets from early destruction to an important extent, but, since a dextrose infusion continued to be necessary, neither CVF nor DS prevented this early loss entirely.

Anticoagulation

aPTT and ACT values were measured after administration of CVF+heparin (Group 2), and DS (Group 3) (Figure 1). The monkeys that were treated with CVF received only one bolus of heparin with the islets, which resulted in an increase of aPTT to a mean of 78sec at 15min, and 56sec at 60min (Figure 1A); their state of anticoagulation was therefore minimal and transient. In monkeys that were treated with DS (initially as an iv bolus, then with the islets, and subsequently as a continuous iv infusion for 6h to 5 days), the ACT was monitored at intervals. The ACT was maintained between 134-235sec during the first 4-6h after Tx (Figure 1B). In Group 3, aPTT was not measured in all cases, but was >150sec after the initial bolus injection of DS. In two monkeys, the continuous DS infusion was associated with

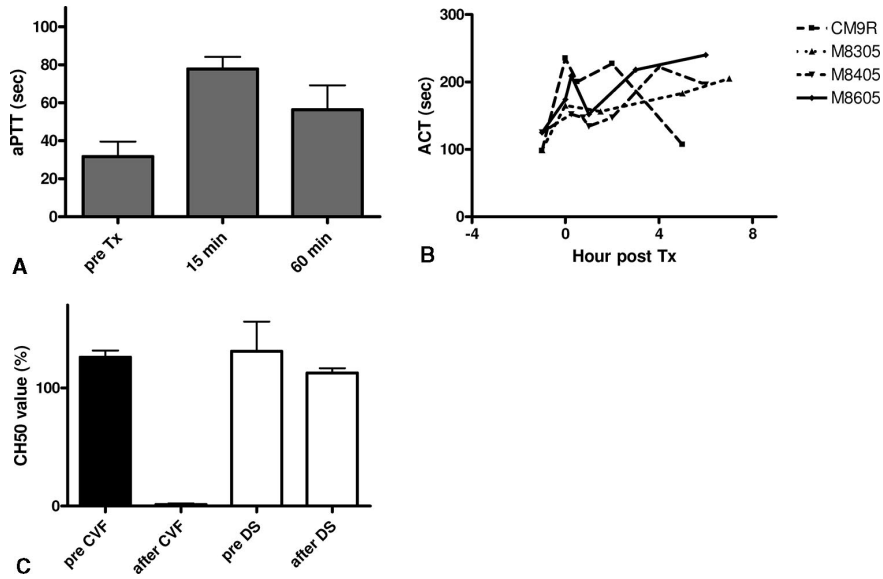

Figure 1:
Anticoagulation and CH-50 values pre-and post-Tx.
(A) aPTT values for monkeys treated with CVF and a single bolus of heparin with the islets (Group 2, n=4). (Pre Tx = before islet Tx; 15 min and 60 min indicate time after islet Tx).
(B) ACT values for monkeys treated with DS (as an iv bolus before islet Tx, + a second bolus with the islets, + by continuous iv infusion after islet Tx) during the first 8h post-Tx (Group 3, n=4). CM9R received DS for only 6h.
(C) CH50 values before and 12h after treatment with CVF (Group 2); and before and 15min after treatment with DS (Group 3) (± standard deviation).

minor superficial bleeding in the abdominal wound, which resulted in discontinuation of the administration of DS at 6h and 30h post-Tx. This complication suggests that the dosage of DS being administered was at a maximum from a safety perspective.

Monitoring of complement (CH50) activity

While CVF was able to completely deplete complement, DS (in the doses used in the present study) only reduced the CH50 value by approximately 15%, indicating complement was active (Figure 1C). There was, however, no obvious difference in the extent of early destruction of the islets, as measured by the amount of dextrose needed to prevent hypoglycemia.

Blood glucose values after islet Tx

The effects of the islet graft on blood glucose, starting 8h after Tx, by which time the

hypoglycemia associated with islet graft destruction was largely completed, and on the need for insulin in the first 5 days are summarized in Figure 2. No immediate effect was seen on the blood glucose level after islet Tx in 2 monkeys (M3604, M3406). Six monkeys showed at least temporary insulin-independence for periods ranging from <1 to >5 days. One monkey (M8405) remained normoglycemic throughout the 5 day period of observation without the need for insulin. Whenever insulin treatment was required (in 5 of the 6 monkeys), in 4 cases this was at a lower level than before Tx. The reduction in insulin requirement to maintain a blood glucose <200mg/dL ranged from 0% to 80%. The blood glucose, and independence from insulin, showed a correlation with the porcine C-peptide value (discussed below) (Figure 5).

Porcine C-peptide values after islet Tx

The normal range of C-peptide in healthy, non-diabetic pigs at our facility is 0.2-0.6ng/mL. In monkeys, after the first 8h, by which time the early destruction of islets and release of insulin and C-peptide had largely occurred, porcine C-peptide levels were measured. Figure 3 indicates these values in relation to the two different immunosuppressive regimens used, and in relation to the agents administered in an attempt to prevent IBMIR.

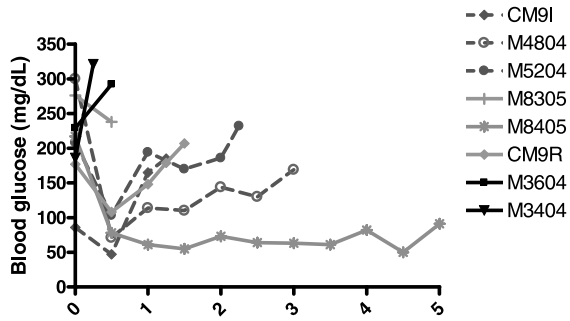

Figure 2:
Blood glucose values of all completely diabetic monkeys (n=8) in the first 5
days after islet Tx, or until insulin administration was started. Monkeys were treated with conventional immunosuppressive drugs (solid black lines; n=2), or with a costimulation blockade-based regimen with CVF and heparin (dashed lines, n=3), or a costimulation blockade-based regimen with DS (grey lines, n=3).

When a conventional immunosuppressive protocol was administered (n=3), in the absence of any therapy directed towards IBMIR (Group 1, n=2, M3604 and M3804), after the Tx of 27,000 or 40,000IEQ/kg, very low C-peptide levels were documented even on day 1 (Figure 3A). In the third animal, when CVF was added to this conventional protocol (n=1, M3404), an

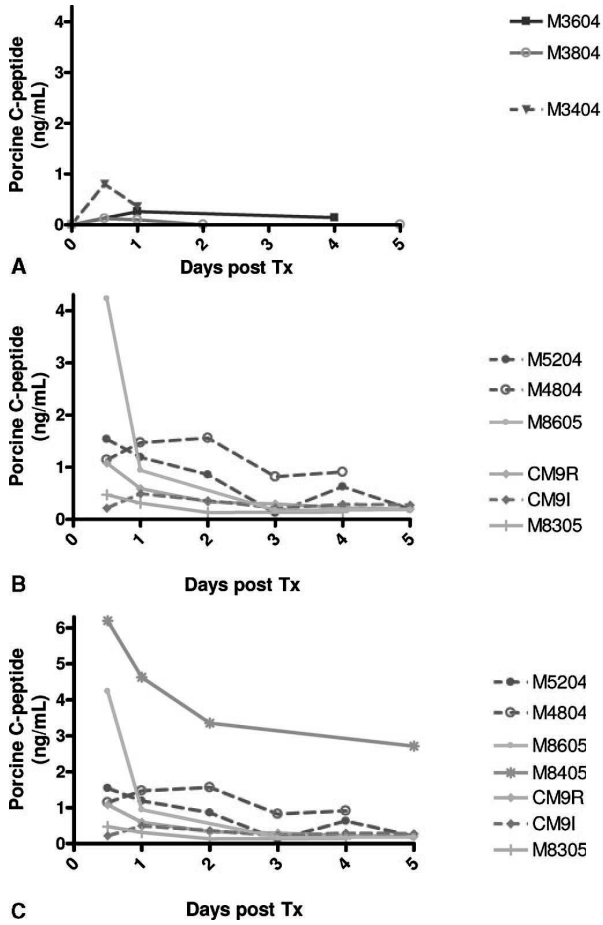

Figure 3:
Porcine C-peptide values in the first days post-Tx.
(A) Monkeys treated with conventional immunosuppression (n=3), with (dotted line, n=1) or without (continuous lines, n=2) CVF. All monkeys received 40,000IEQ/kg intraportally.
(B) Monkeys treated with costimulation blockade and CVF+heparin (Group 2, n=3, dotted lines) or DS (Group 3, n=3, continuous lines) that received 40,000IEQ/kg intraportally (n=6).
(C) Monkeys treated with costimulation blockade and CVF+heparin (Group 2, n=3, dotted lines) or DS (Group 3, n=4, continuous lines) that received either 40,000IEQ/kg (n=6) or 80,000IEQ/kg (n=1, M8405), intraportally. (Note the different scale in C)

increased porcine C-peptide level was documented; however, follow-up of this monkey was possible for only 30h as it required euthanasia for uncontrollable seizures, possibly related to a high level of tacrolimus.

Porcine C-peptide levels were considerably higher in monkeys that were treated with the costimulation blockade regimen (Figure 3B). No clear difference was seen whether treatment was with CVF+heparin (Group 2, n=3, dotted line) or DS (Group 3, n=3, continuous lines). There was, however, a rapid decrease of porcine C-peptide in the single monkey in which diabetes had not been induced (M8605), which also received 40,000IEQ/kg intraportally and the same immunosuppressive regimen.

Using the same immunosuppressive regimen, after the Tx of 80,000IEQ/kg in one monkey (M8405), the levels of C-peptide were higher (initially 6.2ng/mL, reducing to 2.7ng/mL by day 5 [Figure 3C]). Blood glucose levels in this monkey were maintained between 64-91mg/dL without administration of exogenous insulin throughout this 5-day period. On day 1 after the intraportal injection of 80,000IEQ/kg, there was elevation of ALT (217IU/L) and AST (387IU/L), but these had normalized within 4 days. This monkey required euthanasia for a postoperative complication after 7 days, at which time histological examination of the liver showed many viable porcine islets, indicated by staining for insulin (Figure 4).

Subsequent follow-up

Although this report is primarily directed to the effects of the IBMIR seen within the first few days after porcine islet Tx, follow-up was possible in some monkeys. Within the first 14 days, three monkeys had to be euthanized for complications; four other monkeys lost porcine C-peptide production within the first 14 days. Three monkeys were followed up for longer periods. In these, porcine C-peptide production was documented for periods >1 month, but insulin therapy was required in all 3 to maintain the blood glucose <250mg/dL (Figure 5A and B). Despite C-peptide levels that were within, or higher than, the normal range for healthy pigs (0.2-0.6ng/mL), insulin was required to maintain a state of 'normoglycemia'.

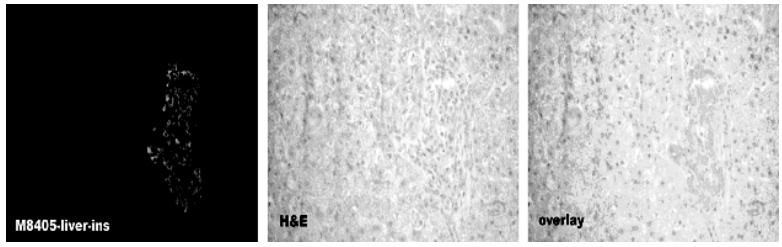

Figure 4:
Microscopic section of the liver in a monkey (M8405) 7 days after the Tx of 80,000IEQ/kg, demonstrating islet grafted tissue stained for insulin (green fluorescence indicates insulin; magnification x20).

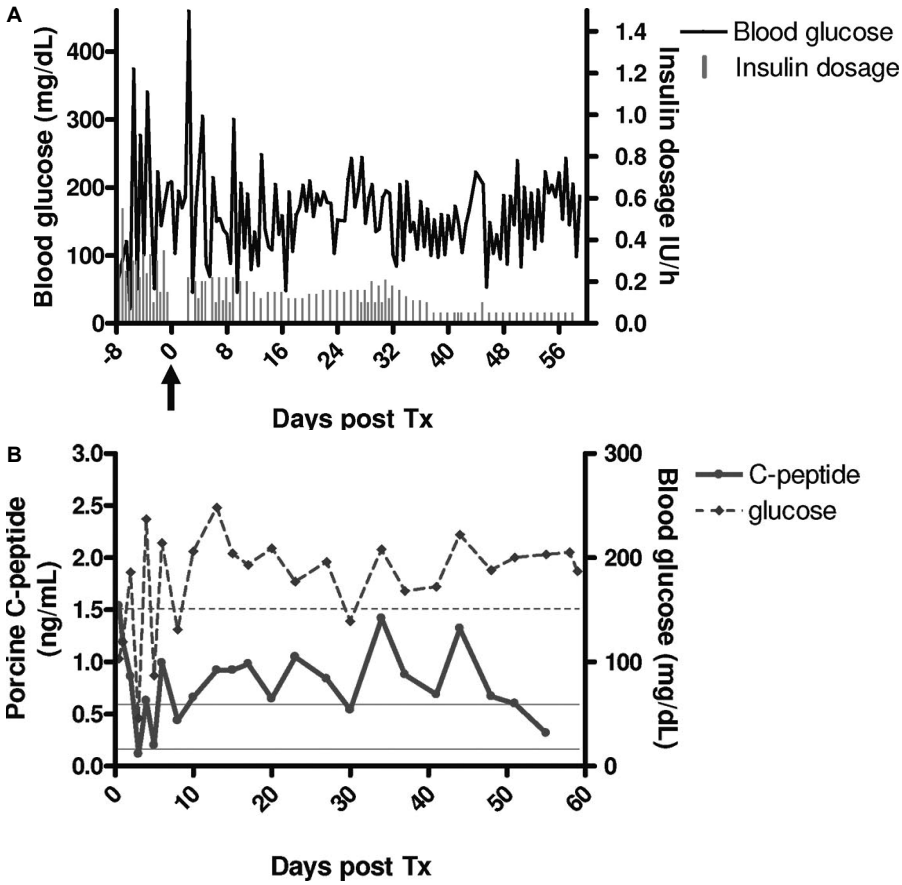

Figure 5:

(A) Blood glucose levels, and insulin requirements, pre- and post-islet Tx in one Group 2 monkey treated with costimulation blockade and CVF+heparin. The arrow indicates the day of islet Tx. Although insulin was required to maintain blood glucose levels <250mg/dL, the insulin requirement was reduced to 25-50% of that pre-Tx.

(B) Porcine C-peptide levels and selected blood glucose levels in the same Group 2 monkey. The two continuous horizontal lines indicate the normal range of porcine C-peptide in healthy non-diabetic pigs, and the single dotted line indicates the lower level of monkey C-peptide in healthy non-diabetic monkeys. Despite normal or high levels of porcine C-peptide, the blood glucose remained relatively high, and the monkey required some insulin (see Figure 5A). The rise in porcine C-peptide in response to a rise in blood glucose indicates survival of viable and functional pig islets.

DISCUSSION

It has been well documented by several groups that large numbers of pig islets are rapidly lost after their intraportal Tx into primate recipients (4, 5, 13), reviewed in (29), and we have confirmed this observation in the present study.

Monkeys in Group 1 (heparin bolus only) and Group 2 (CVF + heparin) demonstrated a low level of anticoagulation for a short period. Group 3 monkeys, treated with DS, had a much higher level of anticoagulation and for a longer period. Therefore, Group 1 represented no significant treatment for IBMIR, Group 2 represented absence of complement activity, and Group 3 represented effective anticoagulation, without significant reduction in complement activity.

In the present study, the extent of the initial injury to the transplanted pig islets from IBMIR was largely estimated by the amount of dextrose required to prevent serious hypoglycemia. Although this is not a perfect primary endpoint, we believe it did provide some indication of the immediate loss of islets from IBMIR. A high dextrose requirement (e.g., in Group 1) correlated with a very short period of graft survival, as measured by blood glucose level, insulin requirement, and the level of porcine C-peptide. Complement depletion with CVF (Group 2) or anticoagulation with DS (Group 3), resulted in an important reduction of early graft loss. Early graft survival was indicated by higher porcine C-peptide levels (Figure 3). However, even with complement depletion or anticoagulation, significant early graft loss could not be prevented completely.

It has been reported that DS alone results in both anticoagulation and inhibition of complement activity (12, 13). Our own observations suggest that, although effective as an anticoagulant, DS was markedly less effective in inhibiting complement activity. Other agents and approaches therefore require to be investigated to prevent IBMIR. For example, a combination of complement depletion (by CVF) and anticoagulation (by DS) may prove more successful, and this is indeed planned as our next study.

The best result was obtained when the number of islets transplanted was increased to 80,000IEQ/kg. Normoglycemia, without the need for exogenous insulin, and high porcine C-peptide levels were documented throughout the 5-day period of observation, indicating that the number of islets transplanted had compensated for the initial loss that resulted from IBMIR[1]. It is perhaps important to note that the normal level of blood glucose in healthy monkeys is 50-70mg/dL; this was the only diabetic monkey in which this relatively low level, or true normoglycemia, was obtained, and this was only achieved when porcine C-peptide

1 We have subsequently repeated this experiment, and have achieved normoglycemia in the absence of exogenous insulin in a monkey that is currently 5 weeks after islet Tx.

levels were between 2.7-6.2ng/mL. In the other monkeys, graft function associated with lower porcine C-peptide levels (0.5-2.0ng/mL), which are actually high levels in healthy pigs (Figure 5), resulted in improvement of the diabetic status, but not in normoglycemia. A potentially important observation is that C-peptide levels in healthy non-diabetic monkeys (>1.5ng/mL in our laboratory) are considerably higher than in healthy non-diabetic pigs (0.2-0.6ng/mL). Peculiar characteristics of monkey metabolism and/or of porcine islets may therefore be relevant to islet function in pig-to-nonhuman primate Tx models. The metabolic differences between pig and primate C-peptide are currently under investigation at our center.

As the number of porcine islets that could be made available for any single diabetic patient will be theoretically limitless, this observation may provide a simple way of overcoming the very considerable barrier raised by IBMIR. However, to provide 80,000IEQ per kg for an 80kg patient might require a total of more than 6 million IEQ, although the number may be significantly fewer since fasting C-peptide levels in healthy humans (1.18+/-0.06ng/mL (30)) are considerably lower than those in monkeys. Pig islet insulin production may therefore sustain normoglycemia more successfully in humans than in monkeys, necessitating the Tx of significantly fewer porcine islets.

Nevertheless, it would clearly be preferable to reduce the percentage loss of islets from IBMIR. Furthermore, although the elevation in liver enzymes after islet Tx in the present study was only transient, there is the potential for portal vein thrombosis or liver injury after the intraportal Tx of very large numbers of islets.

In other studies, it has proved difficult to determine how much loss of islet function is a consequence of IBMIR and how much is a result of rejection. Increasingly, however, the loss from IBMIR is becoming more obvious (3, 31). In our own studies using a costimulation blockade-based regimen, as originally introduced by Buhler et al (32), T cell-mediated acute cellular rejection does not appear to have been a major factor in graft loss, since there has been an absence of cellular infiltration in the liver and of a T cell-dependent elicited antibody response. Yet islet function has still been lost (Figure 5B), suggesting that the islet mass that survived destruction by IBMIR was insufficient to maintain normoglycemia. It may be only when the problem of IBMIR has been resolved that low-dose immunosuppressive therapy will be able to be fully investigated.

GT-KO pigs have not been used previously as sources of islets for Tx into primates. In this present, admittedly very limited, study, as anticipated, we did not find that adult GT-KO pig islets were less susceptible than WT pig islets to early destruction. It has been reported (9) that carefully isolated adult WT islets do not express the Gal epitopes against which primate anti-pig antibodies are largely directed. Our own observations also indicate that endocrine islet cells, including beta cells, do not express Gal epitopes, although Gal-positive cells can be found in small numbers (Figure 6A), most likely as a consequence of imperfect enzymatic

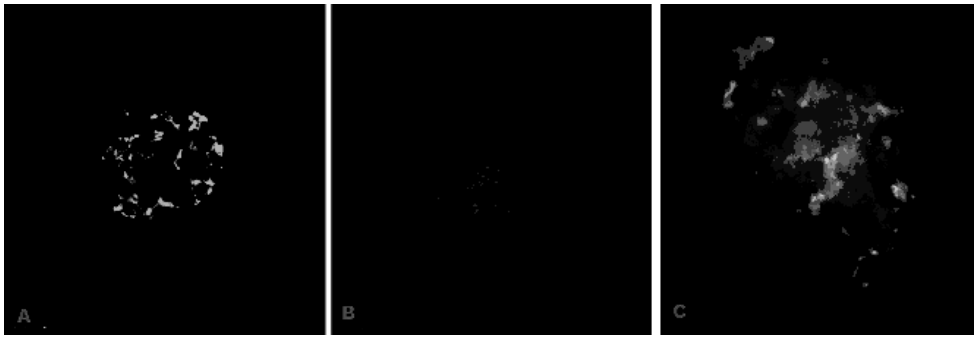

Figure 6:
Isolated islets from **(A)** WT and **(B)** GT-KO pigs, stained with fluorescein-labeled Griffonia simplicifolia 1-isolectin B4 (green), a marker for Gal (magnification x20).
(C) Isolated WT pig islets immunostained for insulin (red) and fluorescein-labeled Griffonia simplicifolia 1-isolectin B4 (green), demonstrating that the insulin-producing beta cells do not stain for Gal (magnification x40).

separation of the exocrine from the endocrine tissues or from the presence of vascular endothelial cells. In contrast, islets from GT-KO pigs show no staining for Gal (Figure 6B). In isolated WT islets, the Gal-stained tissues do not include the insulin-producing beta cells (Figure 6C).

However, early function between the GT-KO and WT islets cannot be strictly compared, as all WT islets were obtained from retired breeding sows whereas the GT-KO islets were not; it has been well-documented that islets from retired breeders have better post-Tx characteristics (15, 16). The outcome may well have been different if neonatal or fetal islets (that express abundant Galα1,3Gal epitopes) had been transplanted. It has been shown in GT-KO mice that transplanted WT neonatal pig islets induce an increase in anti-Galα1,3Gal antibody titer, whereas GT-KO neonatal islets do not (14).

In summary, early loss of pig islets occurred after intraportal Tx in monkeys, even when attempts were made to inhibit IBMIR by CVF+heparin or DS. If clinical islet xenotransplantation is to become a reality, approaches to reduce this initial graft destruction require intensive investigation.

ACKNOWLEDGEMENTS

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Chapter 9
Islet xenotransplantation: Are we really ready for clinical trials?

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ABSTRACT

Four clinical trials of porcine islet transplantation have been reported, and there are verbal reports that clinical trials on much larger scales are continuing in centers in China and Russia. The four reported trials are briefly reviewed and, in the light of the present status of experimental islet xenotransplantation, consideration is given to whether such trials are currently justified. The Ethics Committee of the International Xenotransplantation Association has (i) emphasized the need for encouraging studies in nonhuman primates before clinical trials should be undertaken, (ii) mandatory monitoring for the transfer of porcine microorganisms, and (iii) careful regulation and oversight by recognized bodies. Other aspects of the topic, such as the need for informed consent, are briefly discussed. We conclude that, at the present time, more data documenting convincing efficacy, focused on clinically-applicable immunosuppressive regimens, are needed to justify the initiation of closely-monitored clinical trials. A clinical trial may then be justified even though the potential risk to the patients, and possibly for society, will not be zero.

INTRODUCTION

Of all types of experimental xenotransplantation, islet transplantation is probably the closest to clinical application on a large scale. Over the past several years, progress has been made in the field of experimental islet xenotransplantation in preclinical nonhuman primate models (1), but significant questions remain as to whether progress has been sufficient to move towards clinical trials. Clinical trials of islet xenotransplantation were reported from the Soviet Union in the 1980s and 1990s (reviewed in (2)), and four more recent clinical studies using pig islets have been reported (3-7); these trials are summarized in Table 1. In addition, however, there are verbal reports that clinical trials on much larger scales are continuing in centers in China and Russia.

Clinical Trials Reported To Date

In the early 1990s, Groth et al. (3) in Sweden were the first to report pig islet transplantation into immunosuppressed kidney allotransplant patients with diabetes (n=10) (Table 1). Fetal pig islets were transplanted under the renal capsule (n=2) or intraportally (n=8). Diabetes was not reversed, although patients with intraportally-transplanted islets excreted porcine C-peptide in their urine for varying periods of time, to a maximum of 460 days. It is questionable whether these patients benefited from the transplanted pig islets, since no reduction of insulin requirement was documented. However, the patients were not subjected to unnecessary immunosuppressive treatment, since this treatment was already being administered to protect their kidney allografts.

There have, however, been clinical studies that reported a decrease in exogenous insulin requirement after pig islet transplantation. In New Zealand, Elliott *et al.* (4) transplanted encapsulated neonatal porcine islets into the peritoneal cavity of two patients. One patient was non-immunosuppressed, and the other received immunosuppressive treatment for a prior kidney allotransplant (Table 1). A decrease of insulin requirement was observed and urinary porcine C-peptide excretion was detected in both patients for at least 14 months. HbA1c (glycosylated Hb) levels indicated better long-term control for up to 27 months in the nonimmunosuppressed patient in whom, nine years after islet xenotransplantation, viable encapsulated islets were harvested by laparoscopy; these islets demonstrated insulin release after glucose stimulation *in vitro*. At this time, the patient claimed to continue to experience better glucose control (than pre-transplantation), although this claim was not supported by documented reduced exogenous insulin requirement; an improvement in the patient's HbA1c level was again documented (5).

More recently, Valdes-Gonzales and his colleagues (6) in Mexico reported transplantation of porcine islets, together with porcine Sertoli cells, into steel/Teflon stents placed subcutaneously in non-immunosuppressed adolescent diabetic patients. For various reasons, this trial received considerable criticism in certain medical journals (8, 9). Twelve patients received transplants, of whom 11 received a second transplant after 6 to 9 months, and 4 were again retransplanted after 3 years. In half of the patients, a significant reduction in exogenous insulin requirement was documented for up to 4 years, including two patients who became temporarily insulin-independent (Table 1). Although promising data are available from rodent allotransplantation (10) and xenotransplantation (11) studies with regard to immunoprotection by Sertoli cells, the data available are not conclusive. Recently, it was reported that co-transplantation of neonatal porcine Sertoli cells with islets into diabetic rats did not contribute to graft survival compared to transplantation of islets alone (12). Moreover, the beneficial effect of co-transplantation of porcine Sertoli cells could not be confirmed in a nonhuman primate model of porcine islet transplantation; although in this study no subcutaneous stents were used, no long-term survival was found when neonatal islets, with or without co-cultured Sertoli cells, were transplanted into various sites of nondiabetic nonimmunosuppressed macaques (13).

In 2005, Wang et al. (7) from China reported briefly on the transplantation of neonatal pig islets into the hepatic artery of 20 diabetic patients (Table 1). Various immunosuppressive regimens were administered. All patients who received a steroid-based regimen (n=18) (some of them in combination with tacrolimus and sirolimus) showed a decrease of insulin requirement of 33% to 62% for up to one year, with the presence of porcine C-peptide and without changes in human C-peptide production. Furthermore, HbA1c levels were reported to be normal during this period. The two patients who were transplanted with a steroid-free protocol did not show any improvement in their diabetic status.

Clinical Trials in Relation to the Principles of the Ethics Committee of the International Xenotransplantation Association (IXA)

Physicians and surgeons caring for diabetic patients, particularly those with unstable blood glucose levels who are at risk from sudden hypoglycemic attacks, are clearly stimulated by a desire to help their patients. With the limited supply of human islets, some physicians may wish to proceed with a clinical trial of xenotransplantation, and do not want to wait for confirmatory evidence of a regimen's efficacy from expensive and time-consuming studies of pig islet transplantation in nonhuman primates. However, several aspects of islet xenotransplantation need to be addressed to determine whether clinical trials are currently justified.

All four of the above trials may have been undertaken before the Ethics Committee of the IXA published its principles on clinical trials of xenotransplantation. Without wishing to be critical of these trials, it is of interest to consider whether they would have met the recommendations of the Committee.

The Need for Preliminary Studies in Nonhuman Primates

The principles set out by the Ethics Committee, published first in May, 2003 (14), and subsequently discussed (14, 15), state the need for adequate pre-clinical data to justify a clinical trial, including promising data in a nonhuman primate model. The duration of survival of pig islet grafts in nonhuman primates necessary to justify a clinical trial was not defined by the Committee, as it believed this to be a determination that must take into account the strengths and limitations of the particular studies.

Are studies in nonhuman primates essential before embarking on a clinical trial? They are difficult, expensive, and time-consuming. However, the relative ease with which success can be achieved on occasions in rodent models has not been found to be a good indicator of success in humans, whereas nonhuman primate studies better reflect the hurdles that need to be overcome to achieve clinical success. In view of the possibility of transferring a porcine microorganism to the patient, and perhaps to the community at large, a clinical trial should only be undertaken if there is clear evidence of success in a preclinical nonhuman primate model.

As far as we are aware, no specific preclinical studies in nonhuman primates were carried out, or reported in the peer-reviewed literature, by the above four groups before embarking on clinical trials in the aforementioned human studies. Before the study by Elliott's group, however, Sun et al. had reported normoglycemia after transplantation of encapsulated pig islets into non-immunosuppressed spontaneously-diabetic monkeys (16). Nonhuman primate models of xenotransplantation are not widely available, and the expense and effort involved in these models may preclude this type of research in many centers. Nevertheless, if encouraging data have been obtained from rodent studies, there are centers with the

Table 1:
Experience with clinical pig islet transplantation

First author (reference #)	Source pig islets	Recipients (n)	Site/number of islets (ICC/NPI) (when stated)	Immunosuppressive regimen	Outcome
Groth (3)	WT fetal	*Group A:* 2	*A:* Kidney capsule, 200,000 and 410,000	*A and B:* CyA (n = 10), prednisolone (n = 10)	*A:* No plasma C-pep. Mononuclear and eosinophilic infiltrates at day 21 (on kidney biopsy).
		Group B: 8	*B:* Intraportal, 330,000–1,020,000	ATG (n = 5) 15-deoxyspergualin (n = 5)	*B:* Urine C-pep documented for up to 460 days (n = 4.)
Elliott (4,5)	WT neonatal	2	1 million encapsulated in peritoneal cavity	Non-immunosuppressed (n = 1) CyA, AZA, prednisone (n = 1)	Decreased exogenous insulin requirement (up to 34%), documented urinary C-pep production, and decreased glycosylated Hb for between14–27 months. Nine years post Tx: viable islet cells identified in capsules (n = 1).
Valdes-Gonzalez (6)	WT neonatal + Sertoli cells	12	14,000–21,000/kg subcutaneously, in collagen tubes in steel/teflon stents. Retransplants after 6 months (n = 11) and 3 years (n = 4)	Non-immunosuppressed	Decreased exogenous insulin requirement for up to 4 years (n = 6). No serum C-pep. Glucose-stimulated serum porcine insulin (n = 3).
Wang (7)	WT neonatal	*Group A*: 15	*Groups A, B and C*: Intra-hepatic artery, 5–7 million	*A:* CyA, MMF, prednisolone	*A and B:* Decreased exogenous insulin requirement (33–62%).
		Group B: 3		*B:* OKT-3, tacrolimus, sirolimus, prednisolone	C-pep for 1 year.
		Group C: 2		*C:* CyA, MMF	*C*: No improvement.

ATG = Anti-thymocyte globulin, AZA = azathioprine, C-pep = porcine C-peptide, CyA = cyclosporine, ICC = islet-like cell clusters, MMF = Mycophenolate mofetil, NPI = neonatal pig islet, WT = wild-type.

facilities, expertise, and even the financial support, where such research can be carried out on a collaborative basis. Although difficult, it is no longer a persuasive argument that this type of research was not available to the group considering embarking on a clinical trial.

Monitoring for Porcine Microorganisms

The IXA Ethics Committee addressed the need for monitoring for transfer of porcine infectious agents after transplantation. The issue of the potential transmission of an infectious agent from pig to human continues to be raised, not only with regard to porcine endogenous retrovirus (PERV), but also to other porcine viruses, such as the herpes viruses, e.g., cytomegalovirus and lymphotropic herpes virus (17, 18). To reduce these risks, the Committee recommended that source animals should be obtained from closed colonies from which known and potential pathogens have been excluded. PERV are expressed in porcine islets, though expression does not necessarily mean that there will subsequently be release of virus (19). Cytomegalovirus, but not lymphotropic herpes virus, can readily be eliminated from a pig herd by early-weaning (17).

Furthermore, the possibility of transmission of microorganisms that have not yet been identified, and that could possibly mutate and develop increased virulence, has caused concern, and requires careful consideration whenever a clinical trial is proposed. Monitoring for novel organisms, as far as is conceivably possible, needs to be built into the trial.

In the above trials, islets were not always harvested from pigs in closed 'high-health' status herds (and the definition of 'high-health' varies from center to center). In the Swedish trial, which was undertaken before concern was raised on the potential risks of PERV, the islets were harvested from non-high-health Swedish Landrace pig fetuses. In the New Zealand trial, the islet-source pigs were from a herd of high-health status, although PERV were present. The Mexican trial also used piglets that were bred in New Zealand in a specific pathogen (but not PERV)-free environment. No details on pig source in the Chinese trial were given in the presented abstract.

Furthermore, close monitoring of recipients of any pig xenograft was strongly recommended by the IXA Ethics Committee. Monitoring for transmission of infectious disease has been carried out in the patients in all four trials, if on occasion this has been retrospectively. No adverse infectious event has resulted to date from any of the trials. Follow-up of the patients who have received transplants of porcine islets demonstrated no evidence that they have become infected with PERV (4, 6, 7, 20, 21). However, in some cases, there was little or no evidence of long-term survival of the islets, and it is likely that the PERV were destroyed with the islets. Although the absence of recipient infection with a porcine microorganism is very encouraging, the data remain inconclusive. Our understanding of expert opinion is that, although more investigation is necessary before pig tissue can be declared completely safe, the risk of an adverse effect from PERV is now considered to be low and, indeed, possibly

acceptable (22, 23).

However, as PERV provide only one of many potential infectious risks, every effort must be taken to ensure this risk is minimized before clinical trials should be considered justified. In view of the potential infectious risk to the community, even from microorganisms hitherto unidentified, the perceived risk/benefit ratio must be very carefully considered, more so than with other medical or surgical innovations.

Regulation / Oversight

The IXA Ethics Committee addressed the need to control infectious disease risks through oversight by recognized national bodies (e.g., the Food and Drug Administration [FDA] in the US). As none of the four trials outlined above was carried out in the US, it cannot be expected that any of them were monitored by the FDA, but some form of oversight with regard to infectious organisms would have been highly advisable. Furthermore, the Committee recommended that any future trial should be conducted with detailed oversight by an institutional committee, i.e., be supervised by a recognized official committee at the medical center where the trial is taking place (e.g., an Institutional Review Board in the US).

In this respect, those carrying out the Swedish trial obtained approval by their institutional human ethics committee, although there appeared to be no 'national' oversight, probably because no such authority was in existence at that time. The trial by Elliott *et al.* was carried out with approval of ethical and statutory bodies, although it is not clear at what level these bodies operated. Those carrying out the Mexican trial obtained consent and oversight from institutional and national bodies within Mexico, although no specific 'xenotransplantation authority' exists in that country. Details on the recently reported trial from China are not yet available.

The IXA Ethics Committee also recommended that efforts should be made to work towards international guidelines to ensure monitoring of patients who have received a xenotransplant. To date, no mechanism is in place to monitor (or prevent) a patient with a xenotransplant performed in one country from traveling freely to other countries. (This is important, not only with regard to the xenotransplantation of islets and organs, but also to the xenotransplantation of numerous other cell types, such as fetal sheep cells, that are used as 'therapy' for diverse conditions.) Nonetheless, the recent adoption of resolution WHA57.18 by the 192 countries represented by the World Health Assembly represents a step towards the development of international recommendations and guidelines for well-monitored global application of xenotransplantation practices (24). FDA guidelines on xenotransplantation can be found through their website (www.fda.gov/cber/xap/xap.htm), which also contains links to guidelines from the Council of Europe, EMEA (European Agency for the Evaluation of Medicinal products), and World Health Organization.

Other Considerations

There are several other aspects of clinical trials of xenotransplantation that we have not touched on in this brief commentary, but which must be considered by those planning a clinical trial. Adequate facilities for archiving of tissues and/or blood from both the organ-source pig and the recipient must be available. The potential recipient must have been made aware of the need for life-long monitoring after the transplant, even if the graft fails to function. There are those who believe that procreation of recipients of xenografts should be avoided until the safety of these procedures is assured; this topic must be fully addressed with the potential recipient. In view of these considerations, at this stage in its development, a strong case could be made for xenotransplantation to be performed only in patients in whom the xenograft would be life-saving. With the availability of insulin and islet allotransplantation, patients with diabetes do not generally fall into this category. Although the ideal of immunological tolerance to porcine islets cannot yet be achieved, the immunosuppressive regimen should, at the least, be modest rather than intensive, thus reducing the risks of long-term therapy to the patients.

Finally, the question of whether minors (children and adolescents) should be included in initial clinical trials remains highly controversial. Although their inclusion may well be justified if the procedure will be life-saving, it would be much less justified if this is not the case. In view of the potential risks and possible restrictions on their lifestyles following receiving a xenograft, is it fair to impose these burdens on minors if they are not in a position themselves to give fully-informed legal consent? Space precludes a detailed discussion of informed consent for xenotransplantation, but this important topic has been fully considered by the US Secretary's Advisory Committee on Xenotransplantation (25, 26).

CONCLUSIONS

Although valuable data may be obtained from clinical trials of islet xenotransplantation, and although efforts towards clinical trials should not be unduly impeded, we have to question whether there is sufficient encouraging experimental data in nonhuman primate models to warrant further clinical studies at the present time. Over the past years, some data have become available from pig-to-nonhuman primate studies (1) but, except for a preliminary report by the Minneapolis group on successful survival of pig islets in cynomolgus monkeys for periods of several months using an immunosuppressive regimen that is very unlikely to be clinically applicable (27), there are no studies that support a conclusion that clinical trials are likely to be successful (1).

Although some patients have required less, or even no, insulin (at least temporarily) after pig islet transplantation, and thus the patients have individually benefited from the trial, it remains uncertain whether this was related to improved medical management, e.g.,

meticulous attention to diet, regular monitoring of blood glucose, excellent medical advice and management, etc., rather than to the function of the transplanted islets. Diabetic patients are likely to be more carefully monitored and more attentive to maintaining good control of their own condition when participating in a well-organized clinical trial.

The future may host more clinical islet xenotransplantation studies. If so, in the opinion of the IXA Ethics Committee, it would be mandatory that convincing experimental data in nonhuman primate models as to the efficacy of the approach are available to indicate the likelihood of a successful outcome and to justify exposure to potential risks. Careful monitoring (in collaboration with a recognized national authority) for potential transmission of infectious microorganisms is also surely mandatory. All aspects of the trial should be under the supervision of an institutional (and possibly a national) committee or authority. Such oversight and monitoring will surely not only safeguard the individual patient and the community, but will also increase the likelihood of obtaining valuable data from the trial, even if it is not fully successful in achieving its goals.

At this point in time, more experimental data from nonhuman primate models documenting convincing efficacy, focused on clinically-applicable immunosuppressive regimens, are needed to justify the initiation of clinical trials. These studies should also be designed to provide further data on the safety of the procedure, particularly with regard to the transfer of porcine viruses (including, but not limited to, PERV) to the nonhuman primate recipient of the porcine islets. A carefully-monitored clinical trial may then be justified even though the potential risk to the patients, and possibly for society**,** would not be zero.

What results are required in a nonhuman primate model of porcine islet transplantation to justify progress to a clinical trial remain uncertain. The criteria for a clinical trial are particularly difficult to determine for patients with diabetes, since the disease is not as rapidly fatal as many other conditions for which xenotransplantation offers hope. A strong case could be made for a consensus meeting, and/or the setting up of an expert advisory committee (as convened by the International Society of Heart and Lung Transplantation in 2000 (28)) to determine these criteria.

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Chapter 10
Facilitating physiologic self-regeneration: A step beyond islet cell replacement

P.P.M. Rood, R. Bottino, A.N. Balamurugan, Y. Fan, D.K.C. Cooper, M. Trucco. Pharmaceutical Research, 2006;23(2):227-242

ABSTRACT

Type 1 diabetes (T1D) is an autoimmune disease, the clinical onset of which most frequently presents in children and adolescents who are genetically predisposed. T1D is characterized by specific insulin-producing beta cell destruction. The well-differentiated and specialized islet beta cells seem to physiologically retain the ability to compensate for the cells lost by reproducing themselves, while undifferentiated cell sources may help in generating new ones, even while the autoimmune process takes place. Diabetes clinical onset, i.e., establishment of a detectable, chronic hyperglycemia, occurs at a critical stage when autoimmunity, having acted for a while, supersedes the regenerative effort and reduces the number of beta cells below the physiologic threshold at which the produced insulin becomes insufficient for the body's needs. Clinical solutions aimed at avoiding cumbersome daily insulin administrations by the re-establishment of physiologic insulin production, like whole pancreas or pancreatic islet allotransplantation, are limited by the scarcity of pancreas donors and by the toxic effects of the immunosuppressive drugs administered to prevent rejection. However, new accumulating evidence suggests that, once autoimmunity is abrogated, the endocrine pancreas properties may be sufficient to allow the physiological regenerative process to restore endogenous insulin production, even after the disease has become clinically manifest. Knowledge of these properties of the endocrine pancreas suggests the testing of reliable and clinically-translatable protocols for obliterating autoimmunity, thus allowing the regeneration of the patient's own endocrine cells. The safe induction of an autoimmunity-free status might become a new promising therapy for T1D.

INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease in which autoreactive T cells specifically target and destroy the insulin-producing beta cells of the endocrine pancreas. While the beta cells are selectively destroyed, other non-beta cells, contained in the islets of Langerhans or in the exocrine pancreas, are left more or less intact (1). In the early 1920s, prior to the discovery of insulin, T1D was almost invariably a fatal disease. With the discovery of insulin, the subcutaneous administration of animal-extracted and subsequently human recombinant insulin became the praxis. Since that time, however, studies have shown that only strict control of glycemic levels over the years can significantly reduce – but not completely revert – the incidence of diabetic complications (2-6). As a result, T1D still contributes to the high rate of cardiovascular, microvascular, neuropathic, and retinopathic diseases experienced by our population (7).

Despite marked progress achieved with structural modification, better formulation, and improved mode of administration of insulin, that offer more precise management of glucohomeostasis, diabetics must monitor their blood glucose levels many times a day in

order to determine the appropriate quantity of insulin that needs to be injected. Strict glycemic control entails a sustained effort that a patient must make over many decades, frequently beginning in childhood. Uncontrollable hyperglycemia and/or the peril of hypoglycemia – both potentially life-threatening conditions – impose severe limitations on life-style, as well as health care of patients. Taking into consideration the many variables that may affect glucose regulation in an individual, like hormonal changes, quantity and composition of food intake, different basal metabolism, and even psychological stress, good metabolic control is difficult to achieve even by diligent patients. Thus, insulin replacement therapy alone does not completely protect these individuals from severe consequences, suggesting that more appropriate treatments are needed to get closer to a cure for T1D (8).

Transplantation of the whole pancreas, usually coupled with kidney transplantation, has been considered as one possible therapeutic option. It requires, however, major surgery and, as with any other cell or solid organ allotransplantation, life-long suppression of the immune system of the recipient through administration of immunosuppressive drugs at doses that are frequently associated with toxic effects. This approach has been used almost exclusively in patients with complicated diabetes (9). In the case of simultaneous pancreas and kidney (SPK) transplantation, it has been reported that recipients experience benefits in terms of life expectancy (10). Pancreas transplantation alone (PTA) is proposed for a more limited cohort of diabetic patients: adults with frequent unpredictable hypoglycemic events and overall difficult glycemic control. However, whole pancreas transplantation is not considered as a treatment for diabetic children, due to the severe secondary effects of immunosuppression and a low survival rate when compared with the survival of waiting-list patients receiving conventional insulin therapy, mainly associated with surgical complications and infections (11-16).

In contrast, improved protocols for the transplantation of pancreatic islets have provided new hope for the treatment of T1D (15-17). Islet cells are the defective cell population that diabetic individuals need; their transplantation overcomes the need for management of the acinar tissue of the pancreas and the consequent exocrine secretion typical of whole pancreas implants, which is often the main cause of complications (17). Islet transplantation can be carried out under local anesthesia using a relatively simple and low-risk procedure that a larger number of potential recipients can tolerate safely. In light of the proven feasibility of islet transplantation in animals, and autotransplantations to prevent diabetes in patients in whom the pancreas had to be surgically removed, approximately 750 patients with T1D received allogeneic islet transplants between 1974 and 2000 (15).

The main reason for such a limited number was the rather poor outcome of this intervention until five years ago when the Edmonton group reinvigorated this field by reporting their experience treating 7 patients consecutively who became insulin-independent (18). Insulin-independency was the result of a successful approach that involved the use of a larger islet

mass (obtained combining two to three donor islet transplantations), the employment of freshly-isolated islets (using media devoid of xenogeneic proteins and processed shortly after organ harvesting), and a new steroid-free immunosuppressive 'cocktail' (18). Other transplant centers around the world have now repeated this exciting observation, and have obtained initial success rates of as high as 50% to 80% in terms of providing insulin-independence during the first year (19). However, the initial enthusiasm has been tempered by follow-up studies in which a gradual loss of islet function with time has been observed (20); the percentage of insulin-free patients decreased to less than 10% after 5 years (21). The reasons for immediate or late failure are not completely clear. The procedure of isolation *per se* contributes to impair the quality of the islets, and may constitute the basis for the major problems encountered after grafting in the liver of the diabetic recipient (25-29).

The endocrine pancreas represents approximately 1-2% of the entire pancreatic mass, and constitutes a pellet of tissue of no more than 2-5ml. Such an islet-enriched cell suspension can be implanted via intraportal injection into the recipient's liver, where transplanted islets lodge in the hepatic capillary sinusoids where they are abundantly exposed to portal blood. In light of long-term islet survival in animal studies, the liver has been favored as the best site for islet engraftment in clinical trials. However, the liver site is not ideal for the islets and presents important disadvantages: A thrombotic/inflammatory reaction is elicited when islets come into direct contact with the recipient's blood. The detrimental effects of this instant blood-mediated inflammatory reaction (IBMIR), observed in particular when the islets are transplanted intraportally, seem to provide an additional explanation for the relatively low success rate of clinical islet transplantation (30). Also, steatosis of the hepatocytes surrounding the islet graft has been documented relatively soon after transplantation (23,31).

Finally, the immunosuppressive drugs necessary to avoid recurrence of autoimmunity and allo-rejection are quite toxic, not only to the kidney of the recipient, but also to the transplanted beta cells themselves that eventually die demanding additional transplantations (32-34). The number of available donor organs will continue to limit the number of diabetic patients who can be treated even in the event that transplantation-based approaches, coupled with clinically more acceptable immunosuppressive protocols, prove superior in reestablishment of long-term euglycemia, reduced incidence of T1D complications, as well as overall improved patient health (7,35).

Vigorous research is being performed to improve this situation. Islet allotransplantation has recently been achieved from single deceased obese donors in 8 of 8 T1D recipients (36), and from a single living donor where islets, obtained by distal pancreatectomy, were donated from a mother to her diabetic daughter (37). However, diabetes in this latter case was the iatrogenic result of treatment for chronic pancreatitis; it is well known that islet autotransplantation following total pancreatectomy to treat chronic pancreatitis frequently

results in long-term prevention of diabetes, persisting for more than 13 years post-transplantation (38). Longitudinal studies performed over the next several years will indicate the success of these procedures, and whether they meet the long-term metabolic needs of the transplanted individuals.

These results illustrate the urgent need for exploration of additional avenues in order to realize the goal of curing T1D. For example, regeneration of endocrine pancreas function has been documented after partial pancreatectomy, and in streptozotocin-treated animal models, including mice (39) and rats (40-42), and there are sporadic reports involving spontaneous remission in T1D patients (13,43), as well as evidence for islet neogenesis in non-diabetic obese adult individuals (44). The potential of the pancreas to heal itself seems to be more efficient once autoimmunity is controlled (45). Recent research efforts involving adult stem cells and gene therapy continue to show great potential in animal models. Combining these independent efforts into a unified approach for treating T1D is the challenge awaiting us in our effort to cure this chronic disease.

Type 1 diabetes is an autoimmune disease

In 1993 the paper of Lamperer et al. (46) reported the first unquestionable evidence that also in humans T1D can be transferred by bone marrow (BM) cells. T1D was observed in a woman, aged 29, four years after transplantation of BM from her HLA-identical brother with T1D. At diagnosis of diabetes the recipient was positive for high-titre islet cell antibodies (ICA), whereas she had been ICA negative before transplantation. Chromosomal analyses verified that all circulating leukocytes were of male donor type. This further confirmed the autoimmune nature of the disease fulfilling the first requirement proposed by J.F. Bach (1) to reach this conclusion. The other three criteria are that: 1) the disease course can be slowed or prevented by immunosuppressive therapy; 2) the disease is associated with manifestations of humoral or cell-mediated autoimmunity directed against the target organ; and 3) the disease can be experimentally induced by sensitization against an autoantigen present in the target organ. All these characteristics defining an autoimmune disease find their origin in abnormalities of the physiologic process that brings the T cells to maturation.

In an healthy individual, the maturation of the T cells, coming from cell precursors present in the BM, takes place in the thymus, where they undergo a positive and a negative selection. Both positive selection and negative selection depend on interactions between the T cell receptor (TCR), Major Histocompatibility Complex (MHC) molecule, and antigenic peptide. *Positive selection* occurs as thymic stromal cells bearing MHC molecules, containing self-peptide fragments, engage TCR molecules on the developing thymocytes and direct their continued maturation into functionally mature T-cells. T-cells with ‘useless’ receptors (i.e., those that cannot bind with sufficient affinity the MHC molecule) are not driven to mature and expand, and these cells eventually die in the thymus. *Negative selection* refers to the set of events that specifically eliminate or alternatively ‘anergize’ potentially autoreactive

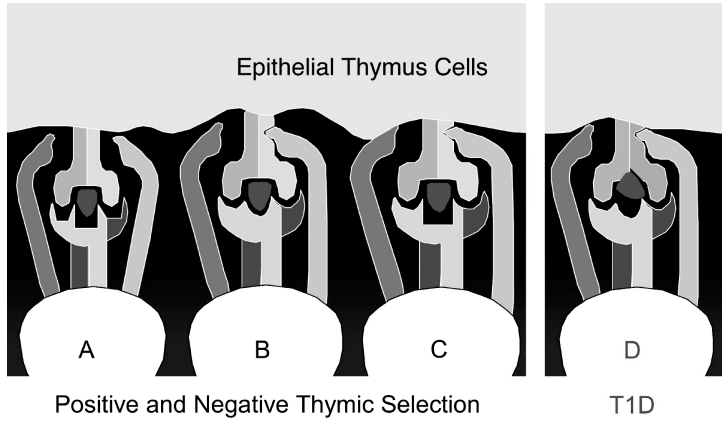

Figure 1:
Theoretical basis of Tian's approach (62) for abrogation of autoimmunity in the NOD mouse.
In the thymus, peptides (in red) from self antigens are presented to the various (A, B, and C) immature double-positive (CD8, in grey, and CD4, lighter gray) T cells via the MHC molecule. When, as for the A cell, the TCR has a very low affinity for the MHC molecule/self-peptide complex the developing T cell does not receive the necessary positive signal to survive. If the affinity between the MHC molecule/self-peptide complex and the TCR is too high, as for the B cell, the T cell undergoes negative selection and dies inside the thymus. In contrast, the T cell shown in C, receives a positive survival signal because of the high-affinity interactions between its TCR and the MHC molecule; an affinity, however, that is not further enhanced by the presence of a self-peptide in its groove, so that the negative selection does not take place. This T cell matures and enters the circulation to protect the body from foreign (non-self) invaders, with which it is able to efficiently interact. In T1D, the D cell binds to an MHC molecule conferring susceptibility to diabetes. The self-peptide is not presented properly. The T cell, then, even if potentially autoreactive (D has the same TCR as B), is not subjected to negative selection, and is free to leave the thymus to circulate in the blood. The approach taken by Tian and colleagues (62) can be illustrated by imagining that the I-Ag7 molecule, able to confer susceptibility to the disease carrying a non-Asp-57 beta chain (in green), is supplemented, in the hematopoietic cells of the NOD mouse, with a non-diabetogenic MHC molecule, i.e., an Asp-57+ beta chain (in yellow), like the one interacting with A, B, or C. Once the cells are returned into the donor, the new MHC molecule (orange and yellow chains) allows the restoration of an efficient negative selection in the thymus (as for B), sufficient to delete autoreactive T cells and consequently to prevent diabetes (108).

cells, thereby inducing 'tolerance' to self (i.e., self-tolerance). During negative selection, factors such as affinity for self-antigen and antigen load likely influence the final outcome of cell death or clonal anergy. Thus, the peripheral T-cell repertoire of each person (including identical twins) is unique and is a consequence of both the random generation of TCRs in the initial unselected thymocyte pool as well as of positive and negative selection events.

More specifically, peptides from antigens of self-tissues are presented to the various immature double-positive $CD4^+$ and $CD8^+$ T cells entering into the thymus (Figure 1). T cells that preferentially bind to MHC class II molecules mature into $CD4^+$ cells. The involvement of $CD4^+$ T cells is unquestionably proven to be of primary importance in the etiology of the disease. Class II molecules are heterodimers composed of an alpha and a beta chain that together form the molecule's antigen-combining site. When the TCR has a very low affinity for the MHC molecule/self-peptide complex (in the cartoon presented in Figure 1, contours of the MHC molecule/self-peptide complex do not fit with the contours of the TCR molecule), the developing T cell does not receive the necessary positive signal to survive and exit the thymus for release into the periphery. However, if the affinity between the MHC molecule/self-peptide complex and the TCR is too high (in the cartoon presented in Figure 1, the contours of the MHC molecule/self-peptide complex fit precisely into the contours of the TCR molecule), the T cell undergoes negative selection and dies inside the thymus. In contrast, the T cell that receives a positive survival signal because of the high-affinity interactions between its TCR and the MHC molecule, but shows an affinity that is not further enhanced by the presence of a self-peptide in its groove, so that the negative selection does not take place, matures and enters the circulation to protect the body from foreign (non-self) invaders, with which it is able to efficiently interact.

The immunological basis of T1D can be found in T cells that bind to an MHC molecule unable to properly present self-peptides. These T cells, then, even if potentially autoreactive are not subjected to negative selection, and are free to leave the thymus to circulate in the blood. T cells that are potentially reactive to self-antigens, but fail to be deleted inside the thymus, are able to attack tissues of the body expressing these same antigens, generating autoimmunity.

In the late eighties, in collaboration with Dr. McDevitt, Stanford University, we were able to map and identify the most influential single hereditary susceptibility factor in T1D: a single amino acid of the beta chain of a class II HLA-DQ histocompatibility molecule (47,48). Although T1D is recognized to be a multigenic disease (49), in humans the principal genetic susceptibility component was proposed to be any allelic form of the HLA-DQ molecule that lacks a charged amino acid at position 57 of its beta chain. Conversely, resistance to disease is associated with the inheritance of HLA-DQ alleles containing a charged amino acid such as aspartic acid, at the same position (Asp-57). Physical explanation of the unusual importance of this particular single amino acid location for the development of the

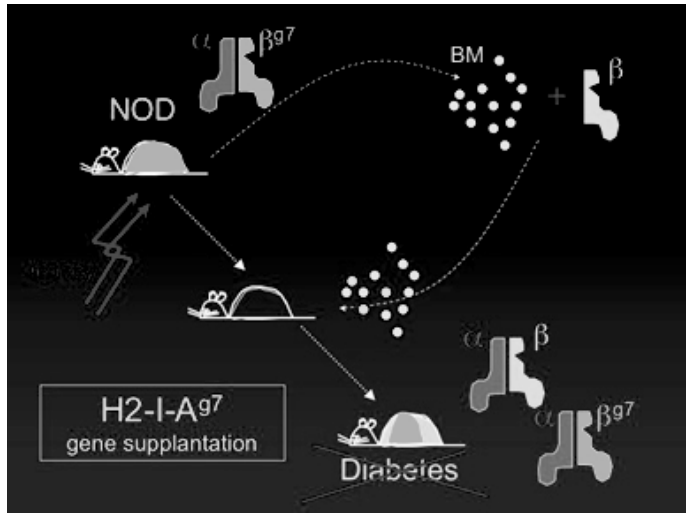

Figure 2:
Schematic representation of Tian's approach (62) for abrogation of autoimmunity in the NOD mouse.
NOD BM stem cells are ex vivo transfected with an H-2 I-A beta chain conferring resistance to diabetes (i.e., an ASP57+ beta chain, in yellow). The transplanted cells are re-infused in the myeloablated donor that will become a chimera carrying the I-Ag7 beta chain (in green) able to confer susceptibility and the resistance beta chain. Since the effect of the resistance molecule is dominant over the other susceptibility molecule, progression to diabetes will be avoided by deleting autoreactive T cells in the repopulated thymus.

autoimmune characteristics of T1D, came with the elucidation of the crystal structure of the HLA-DQ8 molecule, a non-Asp-57 molecule, which confers the highest susceptibility to the disease (50). The most important feature of the susceptibility HLA-DQ8 molecule relevant to diabetes immunology is that its crystal structure is identical to the homologous I-Ag^7 molecule present in the non-obese diabetic (NOD) mouse (51). This strain of mouse spontaneously develops T1D with etiopathogenetic characteristics very similar to the disease in humans. The peptide binding-site of the majority of human HLA-DQ and murine I-A molecules have an Asp-57 that points into the groove. In these allelic forms, Asp-57 forms an electrostatic salt bridge with the arginine in juxtaposition (i.e., in position 76) of the alpha chain of the molecule (Arg-76), which also points into the groove. HLA-DQ8 and I-Ag^7 lack Asp-57 and this variation disrupts the electrostatic interaction, leaving the Arg-76 free to interact with the aqueous environment and with any peptide able to lodge inside the binding groove of the molecule (52,53). The absence of Asp-57 allows the binding of peptides that may not find

appropriate lodging inside other Asp-57$^+$ molecule grooves, and may jeopardize an efficient presentation by the histocompatibility molecule to T-cells because of incorrectly positioned self-peptides. The susceptibility status can be correlated, in immunological terms, with impaired peptide lodging, impaired peptide presentation to T-cells with consequent reduction in positive selection of regulatory T cells or by the impaired negative selection of self-reactive T cells. Indirect evidence supporting these hypotheses derives from transgenic NOD mice that express class II genes other than I-Ag^7, which do not develop diabetes (54-57), and from the fact that transplantation of allogeneic BM from strains that do not spontaneously develop diabetes, also prevents the occurrence of diabetes in NOD mice (58-61).

Recently, instead of approaching the problem using an alloreactive BM transplant, with all its inherent severe contraindications (e.g., GVHD), Tian et al. (62) transfected *ex vivo* the gene encoding a resistant, Asp-57$^+$ beta chain into the BM cells isolated from the diabetes-prone NOD mouse itself (Figure 2). T1D was prevented by the presence of a 'diabetes-resistant' MHC molecule at the surface of hematopoietic stem cells of genetically susceptible (i.e., carrying a 'diabetes-susceptible' allele) NOD mice. The expression of the newly formed diabetes-resistant molecule in the re-infused hematopoietic cells, was sufficient to prevent T1D onset in the NOD mouse even in the presence of the native, diabetogenic non-Asp57, I-Ag^7 molecule. Mechanistically, the authors suggested a model in which a subset of the engineered BM cells – i.e., hematopoietic precursor cells – migrate, populate the thymus and become antigen-presenting cells involved in the negative selection of thymocytes that would otherwise mature into autoreactive T-cells. In fact, diabetes-free NOD mice exhibited neither emergence into the blood stream of T cells capable of responding to putative autoantigens, nor the presence of beta-cell-reactive T cells in the pancreatic islets themselves (i.e., no *insulitis*).

Endocrine pancreas regeneration properties

In both physiologic and pathologic conditions, Lipsett and Finegood (63) attributed the rescue of beta cell mass to increased beta cell replication, increased beta cell size, decreased beta cell death, and the differentiation of possibly existing beta cell progenitors.

In favor of the postulated differentiation of beta cells from progenitor ductal cells is the observation that occasional hormone-positive cells can be found embedded in normal pancreatic ducts (64). The number of these duct-associated endocrine cells increases physiologically as a consequence of severe insulin resistance in obese individuals or during pregnancy (65,66). Similar histological changes are observed under conditions of tissue injury and repair after partial pancreatectomy, duct ligation, cellophane wrapping of the gland, or IFN-γ over-expression driven by the insulin promoter (67-70). Even then, within the ducts, only a small number of cells become insulin-positive. This suggests that, even if some precursor exists, the process of the formation of endocrine cells in tissues other than islets (i.e., neogenesis) is not a common property of the duct epithelium.

However, alpha and beta cells seem to develop from a possibly common, non–hormone-expressing, yet *Pdx1*-positive, precursor (71). These endocrine progenitors may be located in physically close proximity to the duct, but may not actually be components of the ductal epithelium (72). At any rate, these hypothetical precursors are present in extremely small numbers so that lineage analysis becomes very difficult. Considering the lack of known appropriate markers, it becomes even more difficult to quantify their contribution to normal endocrine cell turnover. However, single cell precursors, able to regenerate all kinds of cells present in the islet have been successfully isolated from both the ducts and the islets themselves (73,74). Thus, the working hypothesis of those who are proposing that pancreatic ductal cells can transiently regain a less differentiated state and then become beta cells seems legitimate (75). Increased metabolic demand and tissue injury seem to be efficient in activating this physiologic process of cellular homeostasis (76).

On this basis, it may also be possible to accommodate the results of Dor and colleagues (77) who proposed instead that new beta cells can arise only from the pre-existing beta cells themselves, whether in the normal adult pancreas or after pancreatectomy. As a direct consequence, the number of beta cells should become virtually defined at a certain point in time and, afterwards, glycemia should be controlled only by that defined cellular pool. The data also argue against the possibility of deriving beta cells from adult stem cells *in vivo*. While the results of van der Kooy and Taniguchi (73,74) do not contest the proven yet limited ability of a beta cell to divide, the failure of Dor et al. (77) to observe cells that differentiated from stem or precursor cells might actually be explained by the experiments of Gershengorn's group at the NIH that document the possible transition from epithelial-to-mesenchymal (EMT) cells and vice versa (78). The authors hypothesized that precursor cells could be obtained from insulin-expressing cells that lose their beta cell identity. After expansion, these cells could potentially be redifferentiated into insulin-expressing beta cells via mesenchymal-to-epithelial transition (MET). Indeed, the authors describe some, though rather limited, success in their *Science* paper.

After several days of culture of human islets in serum-containing medium, adherent cells start to migrate out from the islets, and form a monolayer of 'fibroblast-like' cells. Gradually, the population of cells down-regulate insulin and other islet-specific protein expression, but increase mesenchymal progenitor cell marker vimentin production. These cells can be passaged more than 30 times and expanded by a factor of $>10^{12}$ *in vitro*. The authors named the cells human islet-derived precursor cells (hIPCs). Interestingly, when serum was taken away from the culture medium, the hIPCs stopped proliferating and formed aggregates of various size. After two weeks culture of aggregates under serum free condition, islet-specific marker expression, such as insulin and glucagon, were up-regulated 1000 fold. The authors argued that under the serum-containing culture condition, islet cells undergo EMT and become hIPCs, whereas under the serum-free condition, hIPCs undergo MET and start to differentiate back into islet cells.

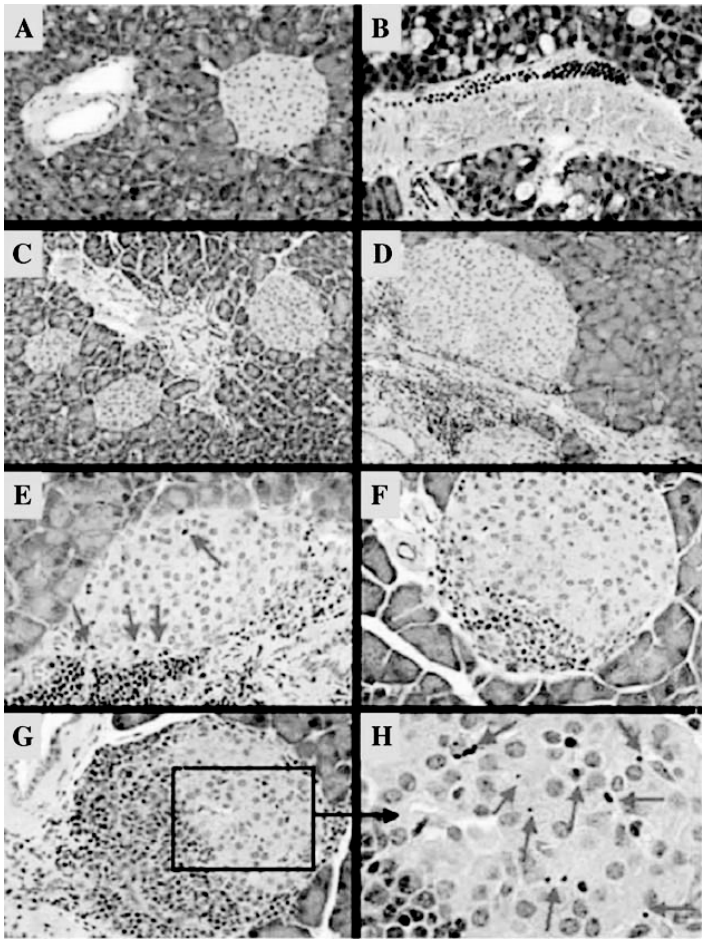

Figure 3:
Scoring of the different stages of destruction of islets of Langerhans during diabetogenesis. Specimens of pancreata from NOD mice of different age were stained with H&E. Magnification for A to G x400, for H x1000. **(A)** Score 0: Normal pancreatic tissue. Neither morphological abnormalities nor mononuclear cell (MNC) infiltration or retention in the pancreatic vessels are present. **(B)** Score 1: MNC vascular retention (yellow arrows). No evident pathological features in pancreatic morphology. **(C)** Score 2: MNC perivascular infiltration (yellow arrows) of the vessels adjacent to the islets; islets maintain a normal morphology. **(D)** Score 3: MNC infiltration in the periphery of the islets (yellow arrows) and in the perivascular area of the adjacent vessels (compare to intact area distant from the islets, green arrows). **(E)** Score 4: The insulitis in the periphery of islets (yellow arrows) is associated with apoptosis (red arrows). **(F)** Score 5: The infiltration of islets by MNC (yellow arrows) is advanced, but not exceeding one third of islet section. **(G)** Score 6: More than one third of the endocrine tissue of the islet is infiltrated by the MNC (yellow arrows). This stage of insulitis is consistently concomitant with extensive apoptosis, presumably of both endocrine and infiltrating cells **(H:** red arrows**)** (60).

If a small number of beta-cells can indeed undergo EMT, and dedifferentiate into precursor cells, in Dor's pulse-and-chase labeling system (77) these cells will still be positively labeled. While Dor and colleagues' explanation inferred a direct replication of beta cells, Gershengorn and colleagues' data (78) suggest that beta-cells can dedifferentiate into precursor cells, which lose beta cell specific marker, while regaining proliferating potential at the same time. Upon proper stimuli, these precursor cells will redifferentiate back into mature beta cells to support islet growth and function.

Further studies are necessary to ultimately define the possible existence and significance of different sources of precursor cells contributing to beta cell regeneration. However, an unconventional type of precursor cell (73,74), possibly located in close proximity and/or inside the endocrine tissue, seems to be present in the pancreas. When metabolic demand increases, these precursors are activated, possibly via various secreted factors that under normal conditions guarantee the cellular homeostasis of the islets of Langerhans.

The balance between autoimmunity and regenerative activity

The physiologic equilibrium between lost and newly generated beta cells can be altered by the action of beta cell–specific, autoreactive T cells (79). Once the killing activity of activated diabetogenic T cell clones overcomes the regenerative compensatory activity of the gland, the number of functional beta cells progressively decrease until they become too few to maintain gluco-homeostasis in the body. After the clinical onset of the disease, even if the regenerative properties of the pancreas remain functional, the continued presence of autoreactive T cells consistently nullifies the reparative effort. Islet cells transplanted from a healthy monozygotic twin were quickly killed by these same autoreactive T cells present in the body of the genetically identical, diabetic recipient twin (80).

The autoimmune response can be successfully averted in the NOD mouse by the successful induction of mixed allogeneic chimerism. The transplantation of BM from a diabetes-resistant animal into a diabetic recipient following a sublethal dose of total body irradiation (TBI), is sufficient to block and eventually also reverse the systematic invasion and inflammation of the islets by autoreactive T cells that results in *insulitis* (58-61).

The allogeneic chimerism induced in pre-diabetic NOD recipients is multilineage and increases with time: four weeks after BM transplantation (BMT) chimerism may reach levels of over 90% (60). In this study, to assess the damage and reparative processes in the pancreata prior to and upon therapeutic intervention, a new morphometric scoring system (called Index N) was utilized; this is comprised of both the degree of insulitis, defined by a very detailed scoring system (Figure 3), and a relative number 'A': the measure of pathology-free area of islet versus whole pancreatic tissue. The need for this new parameter 'A' arose from the observation that in diabetic NOD mice rendered hematopoietic chimerae, a new morphological state of the endocrine pancreas can be recognized. The insulitis-free

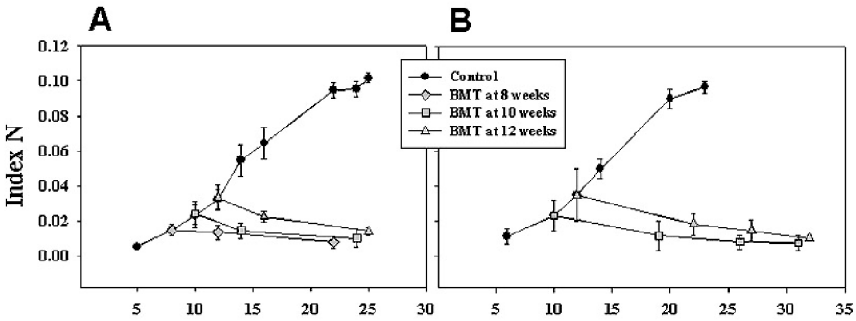

Figure 4:
Chimerism abrogates and reverses destruction of islets of Langerhans in NOD mice prior to the clinical onset of diabetes.
NOD mice (8–12 weeks of age) were rendered hematopoietic chimeras by the administration of T-cell-depleted allogeneic BM into recipients conditioned by lethal **(A)** and nonlethal **(B)** doses of TBI. Pancreata of these chimerae were evaluated for the degree of endocrine pancreas destruction and graded according to Index N. Gray diamonds, squares, and triangles reflect the kinetics of Index N in mice rendered chimeric at 8, 10, and 12 weeks, respectively. Black circles show progression of islet destruction with age evaluated in unmanipulated control NOD mice. This curve was not extended further since untreated animals did not survive long after reaching an Index N over 0.1 (61).

state obtained by the abrogation of the autoimmune process, must be distinguished from the normal, physiologic condition of the pancreas. The islets in the diabetic chimeric NOD mice, although cleared from insulitis, are significantly reduced in size, display an altered morphology and contain cells, none of which has insulin content. In unmanipulated control NOD mice, Index N (insulitis score/A) increased in less than 25 weeks from 0.01 (characteristic of physiological condition) to 0.1 (reflecting the hyperglycemic condition in overtly diabetic mice); at this point the animal dies. In contrast, the chimeric NOD mice were followed to 32 weeks of age, and did not become diabetic (Figure 4). Fourteen weeks after BMT, arrest of the destructive processes and total normalization of Index N were observed in all chimeras subjected to non-lethal doses of TBI. Once normalized, Index N remained at a plateau for 14 weeks (length of observation), confirming that normalization of the structure and function of the insulin-secreting tissue in the endogenous pancreas of chimeric NOD mice was stable and long lasting (61). To prove that the insulin-producing tissue of the endogenous pancreas can undergo a reparative process, direct detection of proliferating (i.e., BrdU-positive) cells can be performed. In the endogenous endocrine pancreas, and in islet allografts of diabetic experimental mice, some proliferating (i.e., BrdU-positive) cells were also positively stained for insulin, revealing the regenerative capacity of the tissue (61).

Normalization of the endocrine pancreas observed in prediabetic NOD mice could also be achieved in these same animals after onset of the overt disease (61). Spontaneously diabetic NOD mice were rendered hematopoietic chimeras by transplanting them with BM from B6-GFP mice (81). The rationale for the use of green fluorescent protein (GFP)-positive BM cells was to track the fate of donor-derived hematopoietic stem cells (HSCs) and to elucidate their possible role in the restoration of the recipient endocrine pancreas. The NOD mice received B6-GFP BM cells along with islet grafts to allow their survival during the time required to reestablish an endogenous insulin production (Figure 5). These animals became

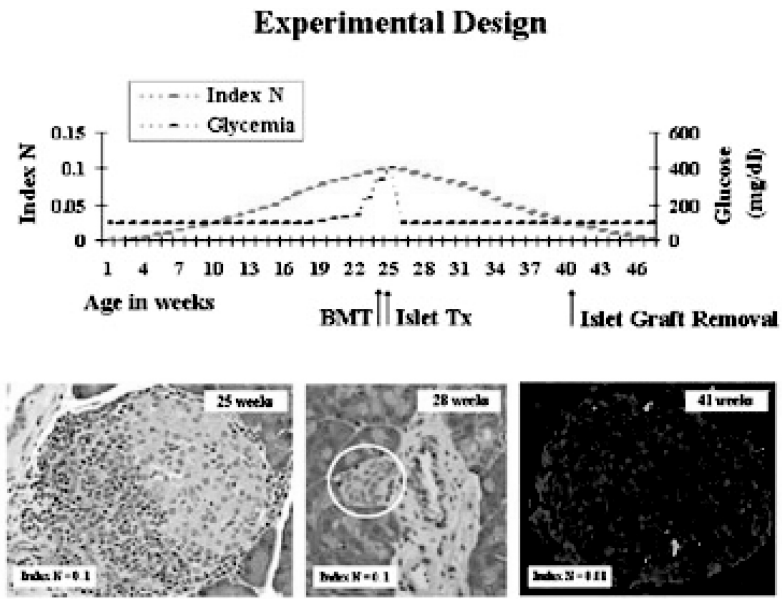

Figure 5:
Schematic representation of the protocol used to test regeneration (or rescue) of the beta cell in diabetic NOD mice.
In NOD mice, the infiltration of autoreactive T cells into the islets of Langerhans (resulting in insulitis) begins at around 4 weeks of age. At 20 to 23 weeks, ~85% of female mice are diabetic, i.e., their glycemia is > 300 mg/dl. When successfully transplanted with bone marrow (BMT) from a non-diabetes-prone donor and hematopoietic chimerism is established, the NOD mouse no longer shows signs of autoimmune activity. However, while there is no more evidence of insulitis in the endogenous pancreas, there is also no sign of insulin production (no red staining). Three to four months after BMT, new insulin-positive cells (shown in red) are present throughout the endogenous pancreas. Thus, when the islets transplanted under the kidney capsule (to maintain euglycemia until regeneration takes place), are removed by nephrectomy, the mice remain non-diabetic (61). For "Index N" morphometric scoring system see ref. 60.

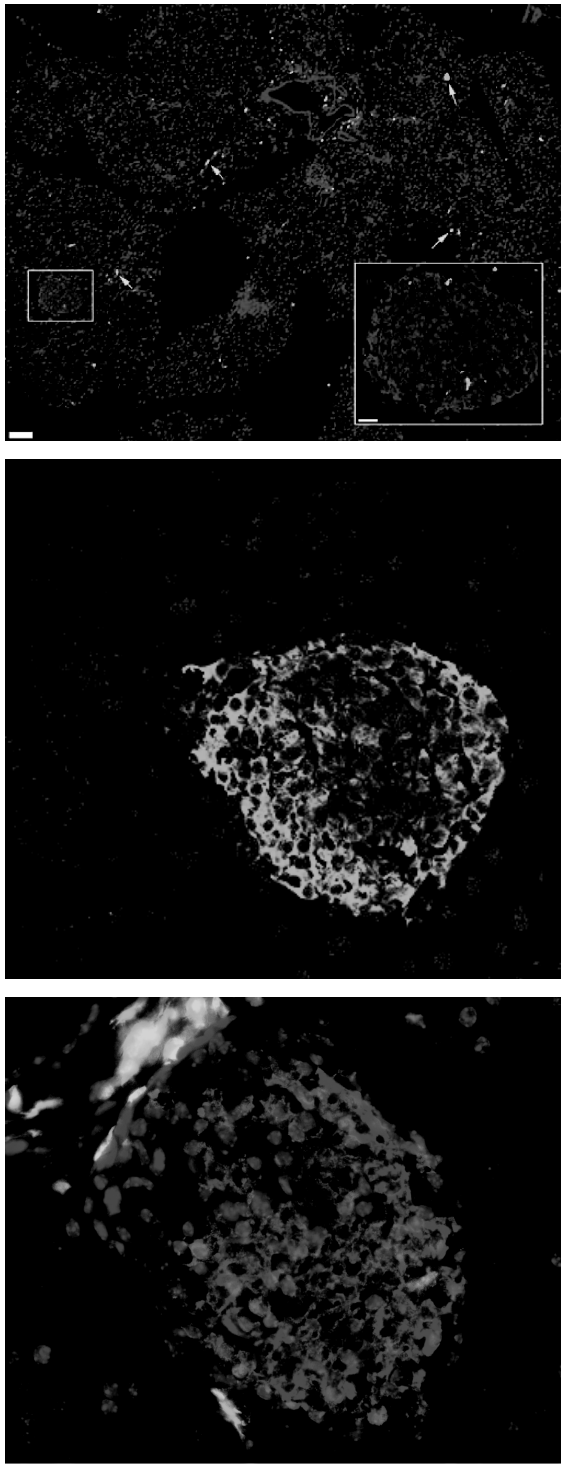

Figure 6:
Regeneration (or rescue) of the endogenous pancreas in a diabetic NOD mouse after obliteration of the autoimmune process via allogeneic BMT.
(A) The regenerated endocrine tissue of a chimeric NOD mouse becomes evident after ~4 months from the BM transplant and takes the shape of cell agglomerates that resemble but are not identical to islets of a non-diabetic animal. Insulin is in red (61). **(B)** Comparison between an islet of Langerhans of a non-diabetic B6 mouse **(A)** with insulin stained green, and a newly formed insulin producing cell agglomerate (in red) in the pancreas of a diabetic NOD mouse treated with BM cells from a non-diabetes-prone, B6-GFP-transgenic donor **(B)**. It is possible to observe that the latter does not have the well organized cell structure of a normal islet and that the majority of the transplanted BM cells (in green) do not directly participate in the regeneration of the endogenous pancreas: there are no double-positive (orange) cells in the newly-formed islets. The donor cells appear to be located close to possibly existing juxta-ductal precursor cells, which may be activated by BM cell–secreted factors (45).

euglycemic within 24 hours following transplantation, and remained so for the period of observation. After surgical removal of islet-graft-bearing kidneys, performed 17–26 weeks after islet transplantation, the mice remained euglycemic. Direct assessment of the insulin content in the islets from the endogenous pancreases that were harvested from euthanized animals 18 days following nephrectomy, revealed insulin-positive beta cells in quantities and morphologies similar to those of the normal mouse pancreas (Figure 6A). Donor-derived GFP-positive cells were detected in the pancreas, but these cells were considered transient circulating, mature blood cells or HSCs not directly involved with the restoration of the endocrine pancreas since insulin$^+$ cells were not GFP$^+$ too (Figure 6B) (45,61). It was actually calculated that, in the cured recipient, insulin-producing cells (that were genetically marked to indicate they are of donor origin) were extremely rare, occurring in 2 out of 100,000 beta cells. These cells may actually be the result of sporadic cell fusion processes (82).

A subject of ongoing debate is whether either or both the transplanted BM and the co-transplanted beta cells are necessary for promoting an efficient regenerative process, independent of their ability to block autoimmunity or preserve euglycemia, respectively. They may, for example, secrete factors that are useful to sustain efficient regeneration. Recent results from the groups of Biason-Lauber, Bouwens, and Rabinovitch are particularly germane to this issue. In the first case, the ability of *PAX4* to favor regeneration of the endocrine pancreas was proven (83). The combinations of epidermal growth factor (EGF) and leukemia inhibitory factor (LIF) (84), or EGF and gastrin (85), were able to convert *in vitro* exocrine or ductal pancreatic cells, respectively, into insulin-producing cells. Additional factors with a possibly useful activity seem to be those used to increase the islet cell mass in transgenic mice or in gene-therapy-treated human islets (86-89). Also, the use of insulin-like growth factor-1 (IGF-1) seems to be useful to promote and/or accelerate islet cell regeneration (90,91), as appears to be the case for glucagon-like peptide 1 (GLP-1), as described by Farilla et al. (92).

In the rat, experimental evidence supports the notion that precursor cells in both endocrine and exocrine tissue are not susceptible to damage by streptozotocin (STZ), i.e., they are not Glut-2-positive. STZ, like alloxan, uses Glut-2 as the receptor to get into the target cells that it eventually kills (40,93,94). Also, even in neonatal STZ-treated rats, a combination of activin A and betacellulin, for example, promoted regeneration of pancreatic beta cells and improved glucose metabolism (41).

Endocrine pancreas regeneration in non-human primates

As previously anticipated, in the year 2000, the clinical possibility of transplanting islets into the livers of diabetic patients was documented; rejection was avoided thanks to an immunosuppressive regimen that reduced the use of tacrolimus and sirolimus, removed the use of steroids, and instead used daclizumab, an antibody against the IL-2 receptor molecule (18). However, as previously discussed, the Edmonton protocol soon showed its

limits. The first limiting factor was the immunosuppressive protocol, which was associated with side effects, and allowed this type of transplantation in certain adult recipients only (95). The second limiting factor was the need for more than one islet donor for each recipient.

To respond to the latter limiting aspect of the Edmonton protocol, some groups looked at a theoretically-unlimited source of transplantable islets. An unlimited source of islets can be found in animals able to produce an insulin very similar to human insulin and in quantities that may satisfy the insulin requirements of an individual of an average body weight. Based on these two parameters, the pig seemed to be the animal of choice. There is only one amino acid difference between human and pig insulin, and the pig is large enough to supply large amounts of donor islets. Pig insulin was successfully used to treat diabetic children for years before recombinant human insulin became available. Also, evidence that pig islets can be used for human transplantation was provided by studies conducted, in particular, in the 1990's in Sweden (96). This possibility was not further explored when it became clear that the alpha1,3 galactose (alpha1,3Gal) epitopes present on pig tissues were the targets of antibodies, normally found in human serum, that are able to quickly reject xenotransplants. This rapid, deleterious reaction is known as 'hyperacute rejection' (HAR). HAR is the major cause of tissue destruction within a few hours after xenotransplantation. The best way to obviate HAR was to work towards the generation of pigs genetically deprived of the activity of the enzyme alpha1,3 galactosyltransferase (alpha1,3GT) and, consequently, free of alpha1,3Gal epitopes at their cell surface (97). In the spring of 2003 (98), our effort of many years (99,100) to generate alpha1,3GT double knockout (DKO) pigs was successfully completed. DKO pigs are better suited as donors for xenotransplantation than their wild-type counterparts since, once their tissues are transplanted into humans or Old World monkeys, they are not targets for a HAR. Adult islet cells from wild-type animals express only low levels of alpha1,3Gal epitopes (101). However, other cells contaminating each preparation used for transplantation do express alpha1,3Gal epitopes at high levels.

Experiments in chemically diabetic (i.e., STZ-treated) monkeys indicated that pig islets can substitute for endogenous islets, producing enough insulin (monitored by pig C-peptide) to control the recipient animal glycemia (102,103). More pertinent to this discussion, however, is the observation that, using a non-calcineurin inhibitor-based immunosuppressive protocol, it has been observed that the monkeys' own pancreatic endocrine tissue is able to regenerate within a period of time similar to that determined for the diabetic mouse. Preliminary studies show that all the insulin$^+$ and Glut-2$^+$ cells disappear in the pancreata of monkeys treated with STZ, but insulin$^+$ and Glut-2$^+$ cells re-appear after 3 to 4 months of treatment. After STZ treatment, the endocrine pancreas of the monkey was no longer able to produce sufficient quantities of insulin to satisfy the need of the animal, which consequently became diabetic. Monkey C-peptide levels remained <0.5ng/ml for the entire duration of all experiments in which conventional immunosuppressive cocktails were used, and the arginine-stimulation-test was always blunted when performed during follow-up. Regenerative properties may

have been overpowered by the effects of the diabetogenic calcineurin inhibitors administered to the monkey. Also, regeneration did not spontaneously take place, at least at a detectable pace, since STZ-treated non-transplanted monkeys continued to need insulin injections after the induction of diabetes. In contrast, in the absence of diabetogenic immunosuppressive agents, using instead an anti-CD154 monoclonal antibody to block the recipient's immune rejection (104), the monkeys transplanted with DKO pig islets not only produced pig C-peptide but eventually (more than three months after STZ treatment) recovered the ability to produce monkey C-peptide. New insulin-producing cells are appearing with time in the monkey's endogenous pancreas eventually forming islet-like conglomerates of cells (Figure 7) (Bottino, et al).

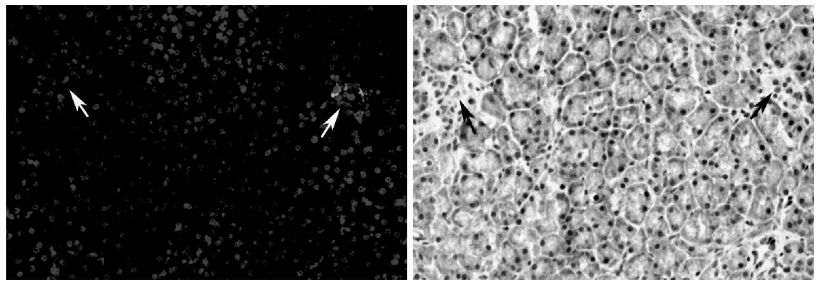

Figure 7:
Newly formed insulin-producing cells in the diabetic monkey.
After 3 to 4 months from STZ injection and diabetes induction, insulin-producing cells are appearing in the monkey endogenous pancreas, eventually forming islet-like conglomerates of insulin-positive cells indicated by the arrows. Immunofluorescence on the left (insulin in green) and H&E on the right, of two consecutive tissue sections (Magnification 20x).

If regeneration can occur not only in rodents but also in the monkeys, we can also expect the endocrine tissue to regenerate in humans once autoimmunity has properly and successfully been abrogated. There is some evidence that supports this expectation.

Endocrine pancreas regeneration in humans

A group from Ulm in Germany recently reported the case of a 13 year old caucasian boy who, after conventional onset of T1D (i.e., the boy presented with a history of polyuria, polydipsia, weight loss, and serum glucose up to ~ 500 mg/dL, glucosuria and ketonuria), needed lower and lower insulin doses over time, allowing his physician to completely discontinue insulin therapy after 11 months (43). The authors also reported that: "Without further treatment, HbA_{1c}, and fasting glucose levels remained normal throughout the entire follow up of currently 4.5 years", and that serum autoantibodies to GAD65, IA-2, insulin, and

ICA “were initially positive but showed a progressive decline or loss during follow-up.” A similar case was recently reported by David Harlan’s group (13).

The main message we draw from all these reports is that within the endocrine pancreas, once the insult of autoimmunity is abrogated, the physiologic process of regeneration can continue efficiently, eventually replenishing the population of insulin-producing cells to a number sufficient to maintain euglycemia, thus curing the diabetic patient. While this process takes place, the recipient’s glycemia must be controlled by additional, independent measures. In rodents, the most commonly used technique has been to transplant into the recipient islets from the same BM donor. However, the successful engraftment of the transplanted BM (necessary to abrogate the autoimmunity) and/or islets (necessary to maintain euglycemia) would have to be promoted and maintained without the use of calcineurin inhibitors that will eventually not only kill the autoreactive T cells of the recipient, but also limit beta cell neogenesis, thereby undermining the success of the transplant (32-34). The use of these diabetogenic immunosuppressive agents may also interfere with the observed rise of regulatory T cells, a possible explanation for the long-lasting immunoregulatory cell–dominant condition observed in cured animals (105). Adoptive transfer experiments in which both diabetogenic lymphocytes from diabetic NOD mice and splenocytes from treated, long-term diabetes-free NOD mice were transplanted into NOD*scid* recipients, with no signs of induction of diabetes, support this hypothesis (106,107).

On these bases it seems that not only in animals, but in humans as well, the abrogation of autoimmunity could allow the physiologic regeneration of insulin-producing beta cells in the host endocrine pancreas, even after the onset of the disease, if a non-diabetogenic immunosuppressive protocol is implemented. These are the premises on which reliable and more clinically-translatable alternatives than allogeneic BMT or allogeneic or xenogeneic pancreatic islet transplantations should be found to cure our young diabetic patients.

Readjusting the efficiency of the first T cell signal

On this basis it seemed useful to extend the previously described experimental protocol proposed by the Harvard group to implement a new one that is more transferable to clinical trials (108): If Tian’s approach (62) to abrogate autoimmunity can facilitate a possible recovery of autologous insulin-producing cells also in the diabetic individual, safe induction of an autoimmunity-free status might become a new promising therapy for T1D. The working hypothesis to be tested considers the use of BM-enriched hematopoietic precursor cells, instead of the non-fractionated BM cell population used by Tian et al., as the recipients of the MHC class II beta chain gene that confers resistance to the disease, to abrogate autoimmunity. Enriched precursors will be more successfully transfected and more easily accepted by the recipient than the total BM cells. Also, differing from Tian’s approach, overtly diabetic (rather than prediabetic) individuals would be treated by the re-infusion of transfected BM-enriched precursors. Autoimmunity will be efficiently abrogated

if the enriched precursors are able to generate the right derivative cells and in sufficient numbers to efficiently repopulate the thymus, by negatively-selecting possibly autoreactive T cell clones and promoting peripheral tolerance mediated by T regulatory cells. In the absence of both autoimmunity and diabetogenic immunosuppressive protocols, by adopting alternative means to correct hyperglycemia, the regenerative property of the autologous endocrine pancreas should repopulate the gland with enough insulin-producing cells to restore euglycemia. Also, to avoid the use of radiation to eliminate the activated T cell clones present in the diabetic patient, as in Tian et al. (62), an antibody-based preconditioning may be used instead. Finally, it should be determined how long after its onset disease reversal remains possible, and the measures to be considered in case this reversal property becomes less efficient with time.

First, all these steps have to be successfully tested in the NOD mouse, as an animal model in which diabetes spontaneously develops as a direct consequence of an autoimmune process, very similar to the one we observe in humans. Then it would be useful to try to reproduce the most promising results in non-human primates. Even if non-human primates do not spontaneously develop autoimmunity, and consequently do not spontaneously develop T1D, this model can be used for testing the safety of the proposed protocol. The concept that a physiologic regenerative capacity may be present in humans will obtain much more support once it has been demonstrated in a closely-related species, such as the monkey.

The proposition of considering the use of this same approach for a possible clinical trial may be complicated by the presence of more than one susceptibility molecule in humans, i.e., not only the HLA-DQ molecule like in the NOD mouse, but also the HLA-DR. However, the efficiency of Tian's gene-based treatment, even in the presence of the native, diabetogenic molecule, may offer solutions also to the problem of dealing with more than one susceptibility molecule (108,109). It is expected that the protective allele will act in an epistatic or dominant manner over the susceptibility allele, also in the case of the DR molecule (109). Thus, to cover all the bases, both a new DQ and a new DR beta chain should be co-transfected into the precursor cells.

For preventing diabetes progression in pre-diabetic NOD animals by transfected BM precursor cell reconstruction, a combination of AutoMACs with Flow Sorter approaches was used to isolate $Sca1^+$, c-Kit^+ and Lin^- BM cells for transplantation into myeloablated recipients. The isolation of BM was performed on 8-week-old F1 NOD/NOD $H2^b$ congenic males. F1 donors were chosen to mimic a syngeneic transplant yet with the possibility of recognizing donor from recipient cells in the reconstituted animal. All NOD female recipients remained alive after sub-lethal radioactive conditioning and showed an evident chimerism in the blood two weeks after receiving enriched BM precursor cells (Fan, unpublished observation).

These preliminary experiments were performed using retroviruses carrying only the GFP gene or the GFP gene plus the gene of the I-A beta chain conferring resistance to the disease. However, in light of a possible clinical trial, the use of a retrovirus for performing a successful resistance beta chain transfection, as proposed by Tian et al. (62), should be avoided since it is associated with the problem of its preferential insertion in positions of the recipient's cell genome that may facilitate the activation of oncogenes, a problem already sadly encountered in human gene therapy treatments (110). It would be safer to utilize phage integrases (111) to guide the stable and irreversible insertion of DNA at specific locations within the genome to satisfy the need for a safe, yet everlasting, synthesis of the beta chain conferring resistance, even in the offspring of the successfully-transfected BM precursor cells.

Some influencing factors of retrovirus activity, besides the preferential position of the insertion in the genome, include the presence of regulatory and bacterial elements in the insertion construct itself, and the number of integrated constructs. Efficient, targeted, single-copy integrations would be helpful for the improvement of transgene efficiency. Phage integrases catalyze site-specific, unidirectional recombination between two short *att* recognition sites. Recombination results in integration when the *att* sites are present on two different DNA molecules, and deletion or inversion when the *att* sites are on the same molecule.

By using the integrases, all the insertion sites can be recognized because of their limited number. Hopefully they will be found in positions that do not alter the activation of any important gene. In the case that possibly dangerous locations are recognized – a case that, statistically, seems to be quite remote – these insertion sites could be obliterated molecularly before transfecting the 'therapeutic' genes. This is theoretically possible by culturing *in vitro* for a few divisions the HSC transfected with the corrective recombinant DNA associated with an antibiotic-resistance gene, so to select only the 'treated' ones. The hope here is that they will remain able to properly repopulate the recipient's BM and thymus, and that they will not result in any additional non-recognized dangerous insertion. However, in practical terms, it can only be concluded that the risk imposed by the use of this new system will dramatically reduce, but not completely exclude, the problems associated with the use of retroviruses.

Irradiation was used in the original experiments of Tian et al. (62) for removing activated T cells from the recipient. However, it would be worthwhile to systematically substitute for irradiation different, antibody-based, immuno-reductive conditioning protocols. Monoclonal antibodies can be tested as an alternative to, or in association with, the use of Thymoglobulin or Campath. Examples are in protocols originally described by Lucienne Chatenoud in which the anti-CD3 antibody was successfully used to prevent the onset of the disease in prediabetic

NOD mice. It was also possible to reverse recent-onset disease by restoring the lost self-tolerance to beta cell antigens in the same strain of mice (105). Another possibility, proposed by Dale Greiner's group in Worcester (112), consists in the use of an anti-CD154 antibody. The potentially dangerous thrombogenic characteristics of some anti-CD154 antibodies may not be too worrisome if the treatment can be, as in this case, very limited in time. A third protocol involved the use of an anti-CD20 monoclonal antibody. A chimeric mouse-human immunoglobulin G with this specificity (Rituximab) has shown efficacy in the treatment of some autoimmune diseases (113). More recently, Rituximab has also been successfully used to improve the outcome of allogeneic HSC (e.g., enriched $CD34^+$) transplantations into patients who suffered chronic graft-versus-host disease (114). Its efficacy in inhibiting the activation of a number of T cell clones in the recipient, by blocking his/her B lymphocyte activity, could be tested here with the aim of pre-conditioning the recipient before performing transfected BM precursor cell autotransplantation.

Reducing the effects of second T cell signals

Thymic or central tolerance must be complemented by the peripheral regulation mediated by cell-antigen-specific T cells. Dendritic cells (DCs) are the primary antigen-presenting cells (APCs) of the immune system that control the activation of naive T cells (115-117). For full activation of naive $CD4^+$ T lymphocytes to occur, a second signal is necessary besides the already described presentation of the antigen to the TCR in the context of the MHC class II molecule present at the surface of the DC. Once properly activated, the T cell up-regulates the CD154 molecule (CD40 ligand) at its cell surface, thereby promoting the initiation of the second signal. The interaction of CD154 with the CD40 molecule results in the up-regulation of CD80 and CD86 at the surface of the APC. Up-regulated CD80 and CD86 will engage the CD28 molecule present on the T cell. The full activation of the T cell is the result of this second signal costimulation.

In the absence of the interactions between CD80, CD86, and CD28, the T cell will either enter a state of functional silence, termed anergy, or will be primed for apoptosis, perhaps in a CD95-CD95L (Fas-FasL)-dependent manner (118). Converging lines of evidence indicate that the phenotype of the DC surface can play an important role in the development of tolerance to self-antigens, and that it can be manipulated to induce allogeneic as well as autoimmune hyporesponsiveness (119). Phenotypically 'immature' DCs, defined by low level expression of cell surface CD40, CD80, and CD86, can elicit host immune suppression in allotransplantation and autoimmunity.

The first use of DC to prevent T1D in NOD mice was documented by Clare-Salzler et al. (120), who demonstrated that transfer of pancreatic lymph node DC derived from 8 to 20 week-old NOD mice into prediabetic NOD mice conferred significant protection from T1D, insulitis, and

adoptive transfer of T1D. The latter was possibly due to the presence of regulatory T cells which attenuated these pathologic processes. More recently, Morel and colleagues have shown prolongation of a diabetes-free state in NOD recipients of BM-derived syngeneic DC (121,122). NOD DC exhibit strong immunostimulatory capacity, underlined by hyperactivation of NF-kappa B (123–125). In fact, the inhibition of NF-kappa B, using short, double-stranded transcriptional decoys, render NOD DC less immunostimulatory. The administration of these engineered DC into NOD prediabetic mice prevents the development of diabetes (126).

A complementary approach is that of engineering DC in a way in which the expression of the costimulatory molecules CD40, CD80, and CD86 only would be suppressed at the cell surface (107). Unlike the intervention on NF-kappa B or the use of anti-CD40L antibodies and CTLA4-Ig, this approach limits the cell population that is targeted, because the treatment is performed *ex vivo* and does not involve systemic dissemination of proteins which, in the instance of CTLA4-Ig and anti-CD40L, have exhibited toxic effects (127,128). The *ex vivo* treatment of BM-derived NOD DCs with a mixture of antisense oligodeoxynucleotides (AS-ODN), targeting the CD40, CD80, and CD86 transcripts, confers specific suppression of the respective cell surface proteins (107). A single injection of these engineered DCs into syngeneic prediabetic female NOD mice significantly delays the incidence of T1D and abolishes any sign of insulitis. More than one injection of AS-ODN-treated DCs maintain the NOD mice diabetes-free indefinitely without affecting the response of T cells to alloantigens. Splenocytes with an increased prevalence of CD4-CD25-CD62L^{+} T cells, from ODN-treated NOD DCs transferred into NOD*scid* recipients, together with splenocytes from a diabetic donor, reduce dramatically the onset of the disease the latter are normally able to induce (107).

The use of AS technology specifically targeting the transcripts of key DC cell surface proteins involved in T cell activation and regulation could be a useful technique to complement central regulation mediated by a newly-populated thymus, and might make T1D cell therapy more efficient (45).

Infusion *in situ* of appropriate factors able to speed up the physiologic regenerative process

The physiologic regenerative potential of the endocrine pancreas seems to be still quite high immediately after (or very close to) the onset of the disease when, in general, there still are some insulin-producing cells able to secrete sufficient insulin to make C-peptide testing possible, i.e., over the minimum level detectable by the appropriate assays. In the mouse and in the monkey (e.g., cynomolgus), the regenerative process seems to take more than 3 months to substitute enough beta cells to allow the detection of an influence on the control of the glycemia of the animal. Even at this point in time, both of these animals do not yet have perfect control of the glycemia, since intravenous glucose tolerance tests (IVGTTs) are still far from normal. However, this result would constitute already a great advantage for the

diabetic patient, even if we do not know for sure whether, at a longer time after clinical onset, the reparative process may still work and at the same speed observed immediately after onset. Preliminary studies in animals seem to indicate that the regenerative process works proportionally more slowly as the time from onset of the disease increases. If eventually the regenerative process ceases to activate, it would be useful to know when that time is, i.e., when the time from onset has become 'too long'.

To help the system to activate the regenerative process, or to speed up a possibly very slow physiologic recovery, even after protracted diabetes insulin therapy, it would be useful to test those different factors that have been proven to be efficient in better achieving this goal (84,85). For other factors, like PAX4 (83), HGF (87), IGF-1 (90,91), or GLP-1 (92), the insulin promoter should be used to construct the cassettes eventually introduced into the vector. In a recent study (Wang et al., unpublished data), the capacity of adeno-associated-virus (AAV)-mediated pancreatic gene transfer was reexamined using the recently available, novel serotypes of AAV coupled with an improved double-stranded AAV vector DNA cassette, which facilitates rapid and stronger transgene expression (129). The advantage of using AAV vectors consists in their lack of immunogenicity, associated with their limited insertion capabilities, that, particularly in dividing cells, eventually leads to loss of expression of the carried gene. It has been shown that robust and relatively long-term gene transfer can be achieved by these vectors in the vast majority, if not all, of the islets. Gene transfer efficiency and vector distribution in the islets are determined by the choice of AAV serotype vectors, as well as by the delivery methods. The pancreatic exocrine acinar cells are highly susceptible to AAV8 infection. To minimize the unwanted gene transfer to non-endocrine pancreatic and non-pancreatic tissues seen after i.p. or i.v. delivery, we explored a topical route by retrograde delivery into the pancreatic duct, similar to the technique ERCP (endoscopic retrograde cholangio-pancreatography) commonly used in patients with pancreatitis. Since the pancreatic beta cell is, by definition, the most important target in gene transfer and therapy for diabetes, we explored the use of the insulin promoter to minimize non-specific transgene expression in unintended cells, such as the pancreatic acinar cells and those beyond the pancreas. As expected, 2 weeks after delivery of AAV8-insulin-promoter-GFP vector in adult mice, strong GFP expression was readily detected exclusively in the islets, but not in the exocrine acinar cells.

CONCLUSION

For decades, efforts have been made to find successful treatments for T1D, such as insulin replacement, pancreas transplantation, and islet transplantation (whether they be allo or xeno). Despite progress in the field of transplantation, this has not yet resulted in a permanent solution.

Rodent studies have given us hope for a new direction: regeneration of the patient's own beta cells. Preliminary studies in primates support anecdotal examples suggesting that beta cell regeneration might be possible also in humans. If abrogation of autoimmunity can be safely achieved in a diabetic patient with an autotransplant of precursor cells transfected with HLA class II beta chain genes conferring resistance to the disease, while correcting his/her hyperglycemia using conventional insulin administration or an islet allotransplant, 'Nature' will be left to heal the rest. It should also be possible to speed up the natural process of healing by endoscopic retrograde intraductal delivery of factors known to promote beta cell regeneration. Should this approach work satisfactorily, our young patients will be cured for good, without the need for long-term drug therapies associated with the known troublesome consequences.

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Chapter 11
Summary and discussion

The ultimate goal is to find a cure or permanent treatment for type-1 diabetes, which would prevent its notorious long-term complications and abrogate the restrictions that insulin therapy constitutes for daily life. Pancreas transplantation and islet allotransplantation (using a human donor organ) are potential solutions, although not ideal for reasons such as surgical complications and organ shortage.

A potential solution to the problem of organ shortage can be found in xenotransplantation, using the pig as the source of islets. With the production of pigs homozygous for α1,3-galactosyltransferase gene-knockout (GT-KO), a new era has started in the field of pig to primate xenotransplantation. Clinical trials cannot be started without appropriate validation of results obtained from rodent studies in a preclinical model, which necessitates nonhuman primate studies.

This dissertation began with a review of prior experience with pig-to-nonhuman primate islet transplantation (**Chapter 2**). The type of porcine islets used was discussed, i.e., fetal, neonatal and adult islets. It would seem that adult porcine islets have the best potential, from both logistic and immunologic perspectives, to reverse diabetes in primates. Islet isolation techniques have improved sufficiently so that large numbers of good-quality adult porcine islets can be obtained. Models for diabetes in nonhuman primates were described. Refinements in the induction and maintenance of streptozotocin (STZ)-induced diabetes in nonhuman primates provided a useful experimental model, although it remains uncertain whether STZ induces irreversible diabetes. However, progress in extending survival of transplanted pig islets in nonhuman primates has been slow, although encouraging results have recently been reported (1, 2).

With the relative plethora of established and novel immunosuppressive agents now available, one major problem will be to determine the optimal combination. Particularly in the treatment of a disease such as diabetes, that is not rapidly fatal, there is a need to establish clinically-applicable immunosuppressive regimens that are not associated with life-threatening side effects. There will almost certainly be a move away from calcineurin inhibitors, that are diabetogenic, towards newer agents, such as leflunomide, and biological agents that inhibit costimulation. Experimental models have been established in which these novel agents can be investigated. However, as one reviews the literature, questions remain, and details of these models need to be adequately addressed.

ANTI-NONGAL ANTIBODIES

Transplantation of a wild-type (WT) pig graft into a nonimmunosuppressed primate usually results in hyperacute rejection within minutes to hours (3, 4). The primary role of anti-Gal antibodies in this response has been established by several groups (5-8). The problem

of anti-Gal antibodies has recently been overcome by the breeding of pigs homozygous for α1,3-galactosyltransferase gene-knockout (GT-KO) (9, 10). However, although the transplantation of an organ from a GT-KO pig into an immunosuppressed nonhuman primate allows extended graft survival (11), the graft eventually fails from the development of a thrombotic microangiopathy that may be a form of delayed antibody-mediated rejection. The focal deposition of IgM, IgG, and C4d on the graft suggests that a component of this process involves the binding of anti-nonGal antibodies to the pig vascular endothelial cells. These antibodies could be either preformed or elicited.

In addition to this potential role in the rejection of GT-KO pig organs, anti-nonGal antibodies may also be important following adult pig islet transplantation in primates. As adult pig islets are known to express little or no Gal (12-15), anti-nonGal antibodies are likely to be involved in any antibody-mediated rejection process (16-18).

Anti-nonGal antibodies in baboons

In **Chapter 3** we investigated the incidence of preformed anti-nonGal antibodies in the serum of naïve baboons, and we assessed whether these antibodies are cytotoxic to pig peripheral blood mononuclear cells (PBMC) lacking the Gal epitope. The results were compared with reactivity of anti-pig antibodies to WT pig PBMC.

Whereas all but one of 56 baboons had preformed antibodies, mainly IgM, to WT pig PBMC, less than half had such antibodies to GT-KO PBMC. Moreover, the cytotoxicity associated with anti-nonGal antibodies alone (to GT-KO PBMC) was significantly weaker than that associated with combined anti-Gal+anti-nonGal antibodies (to WT PBMC). Our data suggest that GT-KO pigs are preferable to WT pigs as sources of organs for transplantation into baboons. Nevertheless, anti-nonGal antibodies initiated a cytotoxic response; in vivo, this would be expected to jeopardize long-term viability of a pig organ graft.

The capacity of elicited antibodies, collected from the sera of two baboons exposed to GT-KO pig hearts, to bind to GT-KO PBMC, and to lyse those cells, was also assessed. Immunoglobulins, predominantly of the IgG isotype and specific for undetermined nonGal antigens, reached high levels post-transplantation, and were associated with a significant increase in cell lysis. The timing of the elicited antibody response (beginning by day 7) was similar to that seen previously when baboons were exposed to WT pig antigens, but the amplitude of the response would appear to be reduced (19, 20).

We found no evidence of continued expression of Gal in the GT-KO pigs, e.g., as a result of iGb3 synthetase activity, as has been suggested by others (21, 22) since, after exposure to GT-KO pig hearts, and despite sensitization to nonGal antigens, there was no increase in the level of anti-Gal antibodies. Previous in vitro studies involving WT cells and either immunoadsorption of primate sera or treatment of the cells with a galactosidase have

suggested that antibodies to nonGal antigens represent only 10-30% of anti-pig IgM and 20-50% of anti-pig IgG antibodies in naïve sera (reviewed in (23)). The data observed using GT-KO PBMC in the present study would largely agree with these estimates, but would suggest that anti-nonGal antibodies may represent a greater proportion of anti-pig antibodies in some baboons.

Anti-nonGal antibodies in humans

In **Chapter 4** we reported on the incidence of preformed anti-nonGal antibodies in the serum of healthy and allosensitized humans, and we assessed whether these antibodies are cytotoxic to pig PBMC lacking the Gal epitope. The results were compared with reactivity of anti-pig antibodies to WT pig PBMC.

Whereas all healthy and allosensitized humans had preformed antibodies to WT pig PBMC, in approximately one-third antibodies to GT-KO PBMC were not detectable. Moreover, the cytotoxicity associated with anti-nonGal antibodies was significantly weaker than that associated with anti-Gal antibodies, corresponding to our findings in baboons (Chapter 3). Whereas reactivity with WT pig cells is clearly due to the binding of both anti-Gal and anti-nonGal antibodies, reactivity with GT-KO cells is solely due to the presence of anti-nonGal antibodies. However, although the absence of the Gal epitope led to a significant reduction in both the prevalence and severity of complement-induced lysis, anti-nonGal antibodies nonetheless can initiate a cytotoxic response which, in vivo, could affect long-term viability of a pig graft.

An important finding from this study is the documentation that sera from allosensitized subjects did not show a higher level of binding of preformed antibodies (IgM or IgG) to nonGal antigens than did sera from unsensitized or healthy subjects, nor increased cytotoxicity. Previously, some reports suggested that sera from allosensitized subjects might be more cytotoxic to unmodified pig cells than sera from unsensitized subjects, although other reports did not confirm this observation (reviewed in (24)). Recently, it has been confirmed by others that humans previously exposed and sensitized to alloantigens are at no greater risk for rejecting a GT-KO pig xenograft by an antibody-mediated mechanism (25). Highly allosensitized patients frequently have difficulty in obtaining a human donor organ against which they do not have anti-HLA antibodies. This group of patients may therefore be among those first considered for xenotransplantation.

Similar to our findings in baboons, our study suggests that GT-KO pigs are more suitable than WT pigs as sources of organs or tissue for transplantation into humans, since reactivity with preformed antibodies in human serum was significantly reduced. However, unidentified nonGal epitopes on the pig vascular endothelium could still pose a threat to the viability of a porcine graft transplanted into a human.

Acute humoral xenograft rejection (AHXR) associated with the presence of preformed or elicited anti-nonGal antibodies might therefore be addressed by further modification of the pig to reduce the expression of nonGal antigens. The identification of nonGal specificities would therefore be an important step forward. However, the nature of the nonGal antigens expressed on GT-KO pig vascular endothelium or PBMC is currently uncertain, although several carbohydrates are candidates (26, 27). It has been demonstrated that there is some rearrangement of the carbohydrate pattern of the vascular endothelium in mice after GT-KO (28), and neoantigens might be exposed, to which some preformed antibodies may be directed. N-glycolylneuraminic acid epitopes, so-called Hanganutziu-Deicher antigens, are widely expressed on the endothelial cells of all mammals except humans, and are considered to be potential porcine targets for preformed and elicited anti-nonGal antibodies in humans (29-32), but not in baboons, which express these epitopes. It is equally likely that the targets for elicited anti-nonGal antibodies are proteins, rather than carbohydrates.

If there are multiple nonGal epitopes against which humans have preformed antibodies, then gene-knockout may not be a feasible solution. However, the lower level of endothelial cell activation and cytotoxicity associated with anti-nonGal antibodies, compared with anti-Gal antibodies, may allow protection by the presence of one or more human complement-regulatory proteins when combined with a GT-KO pig genotype. Although transgenic pigs expressing human decay-accelerating factor or other complement-regulatory proteins were incompletely protected against anti-Gal antibodies (33, 34), this approach may prove more successful against the weaker anti-nonGal antibodies.

There is increasing success in achieving a state of accommodation after allografting across the ABO blood group barrier or when there is allosensitization associated with anti-HLA antibodies (35). Such approaches, involving procedures such as pre- and post-transplant plasmapheresis and the administration of intravenous immunoglobulin (36), although not effective in inducing accommodation to a WT pig xenograft (37-39), may possibly prove more successful in the presence of the lower levels of anti-nonGal antibody. To our knowledge, this approach has not yet been tested in the GT-KO pig-to-nonhuman primate model.

Another potential way to avoid rejection caused by anti-nonGal antibodies, is to transplant porcine tissue or organs before anti-nonGal antibodies have developed during infancy. This is described in Chapter 6, and will be discussed below.

Anti-nonGal antibodies in monkeys

Chapter 5 describes the presence of anti-nonGal antibodies in monkey sera, and compares the levels of anti-nonGal antibodies between monkeys, baboons and humans. The incidence of binding of monkey serum IgM and IgG isotypes to PBMC from GT-KO pigs was significantly less when compared to binding to WT PBMC. However, we demonstrated that 76% of the tested monkey sera caused some lysis of GT-KO PBMC (compared to 100% lysis of

WT PBMC), although, the extent of cell lysis associated with anti-nonGal antibodies was significantly less.

Even though this confirms the potential advantage of using GT-KO donor pigs for transplantation into cynomolgus monkeys (as we described for baboons and humans), nevertheless approximately three quarters of the monkeys had IgM with or without IgG binding to GT-KO PBMC. Only 43% of human and 32% of baboon sera were demonstrated to have IgM binding to GT-KO PBMC, which is significantly less than the 76% IgM binding of cynomolgus monkey sera ($p<0.01$). Both human and monkey sera showed an incidence of 67% IgG binding to GT-KO PBMC, compared to an incidence of only 9% of baboon IgG.

Furthermore, whereas approximately 50% of both human and baboon sera demonstrated cytotoxic antibodies against GT-KO PBMC, the incidence is 76% in monkey sera. Because of the cytotoxicity associated with these anti-nonGal antibodies, they are likely to be associated with the development of AHXR. These results suggest that monkeys may have stronger antibody-mediated cytotoxic responses to GT-KO pig grafts than humans and baboons.

To our knowledge, there are no reports of transplantation of GT-KO organs or cells in cynomolgus monkeys. However, there is evidence that anti-nonGal antibodies are associated with AHXR in cynomolgus monkeys; Lam et al. demonstrated that, when hDAF-transgenic pig hearts were transplanted into cynomolgus monkeys under soluble Gal saccharide-based therapy (to adsorb or 'neutralize' anti-Gal antibodies), anti-nonGal antibody levels increased in animals that rejected their grafts (40).

It has sometimes been considered that the baboon is a more difficult preclinical model of xenotransplantation than the macaque monkey. Our data suggest that antibody-mediated xenograft rejection of GT-KO pig organs or cells may be more vigorous in cynomolgus monkeys than in baboons or humans. With regard to the cytotoxicity associated with anti-nonGal antibody binding, the baboon may be closer to the human than the cynomolgus monkey, and therefore may be the preferred experimental recipient of GT-KO pig grafts.

Anti-nonGal antibodies in infants

Natural anti-pig antibodies develop during infancy (41-46), a finding confirmed by our study (**Chapter 6**). However, we were the first to investigate the development of anti-nonGal antibodies in infant baboons and humans, and to demonstrate the correlation between age and antibody production. Our data suggest that both baboons and humans develop cytotoxic anti-pig IgM early during the first year, the level of which steadily increases with age during the first year, but this IgM is largely directed against Gal targets. Cytotoxic anti-nonGal antibodies develop later, and generally remain at a minimal level. This increase in anti-pig antibodies with age during the first year is consistent with that of anti-A/B antibodies.

As we described in adults (47), the incidence and extent of binding of infant baboon and human serum IgM and IgG to PBMC from GT-KO pigs was significantly less when compared to binding to WT PBMC. The associated lysis of WT cells was higher in baboons than in humans, suggesting that antibody-mediated rejection of a transplanted WT pig organ may be more problematic in the infant baboon than in the infant human. The increase in anti-WT cytotoxic antibodies was confirmed by sequential measurement of sera from the same baboon. More importantly, the cytotoxicity of infant human anti-nonGal antibodies did not increase with age and was absent in a significant number of cases. This allows a 'window of opportunity' during which a GT-KO pig organ could be transplanted into a human infant without the risk of rejection associated with the presence of natural anti-nonGal cytotoxic antibodies. As occurs in the case of ABO-incompatible heart allotransplantation in human infants (48, 49), B cell tolerance to carbohydrate antigens may develop when a GT-KO pig heart is transplanted into a primate recipient that has not yet developed anti-nonGal antibodies.

Immune responses to other xenoantigens (against which no natural antibodies are present) would need to be suppressed, but it is not definitively known whether immunosuppressive therapy would need to be continued throughout the life of the recipient. Data from Bailey and his colleagues (50, 51) suggest that the immune response to an allograft in neonates and infants is rather less vigorous than in later life, and West et al (49) have drawn attention to the relative 'plasticity' of the neonatal immune system. It would be of great clinical interest to study whether, in this age group, the prevention of a T cell response for a period of time might result in T cell tolerance.

Baboons would generally appear to develop anti-A/B antibodies at approximately 4 months of life, which is similar to the time of their development in humans (49). A previous study using adult human and baboon sera showed no statistically significant correlation between the presence of anti-Gal antibodies and anti-A/B antibodies (52), which is confirmed for IgG antibodies by our findings. However, we found a correlation between the presence of anti-A/B antibodies and anti-WT IgM in infant baboons, possibly since both levels are known to increase during the first year of life.

Our data suggest that GT-KO organ transplants could be carried out in early infancy in human recipients in the complete or relative absence of cytotoxic anti-nonGal antibodies. If immunosuppressive therapy were administered to suppress a T cell-mediated elicited antibody response, xenograft rejection resulting from anti-nonGal antibodies would be prevented (although other potential problems may still arise (53)). Based on clinical experience of heart allotransplantation in infant humans across the ABO barrier, it is possible that B cell tolerance to pig carbohydrate antigens might develop. Future studies should focus on testing this in vivo, by implanting tissue/organs from GT-KO pigs into infant baboons.

PIG-TO-MONKEY ISLET TRANSPLANTATION

Induction of diabetes

Chapter 7 presents the first study to describe the safe induction of diabetes in nonhuman primates with high-dose Zanosar STZ, resulting in undetectable (fasting and stimulated) primate C-peptide. Zanosar STZ is used in patients with metastatic islet cell carcinoma, and may have fewer side effects than the widely used Sigma STZ, perhaps because of greater purity and less variability.

There has been discussion with regard to the optimal dose of STZ in nonhuman primates. Pitkin et al. and Litwak et al. showed that a single dose of 30mg/kg is insufficient to induce complete diabetes (54, 55). Larger doses of 100-150mg/kg were found sufficient (56-58), but were associated with more side effects and complications (54, 57-60). In contrast, Koulmanda et al. reported that 55mg/kg was sufficient to consistently induce diabetes in cynomolgus monkeys (56), but others have been less successful with this dosage (54, 60-62), with C-peptide production still detectable (in the range of 0.6 to 0.9ng/mL). Wijkstrom et al. suggested the dose should be based on body surface area (1250mg/m2), rather than body weight (58).

The islets of younger nonhuman primates may be more resistant to destruction, and this may account for the varying STZ dosages required to induce diabetes (56, 62). Furthermore, since STZ has a half-life of only 10 min (56), it seems reasonable to anticipate that higher dosages are necessary if it is not administered rapidly as a bolus.

The correlation between the dose of STZ and the development of renal and hepatic damage is well-documented. This is demonstrated by an increase in blood urea nitrogen, serum creatinine, and/or hepatic enzymes, or by histological injury (56, 57, 59, 60). It is therefore of importance to find the optimum dose to successfully induce diabetes with minimal or no risk of side-effects.

We have demonstrated that diabetes can be consistently induced after high-dose STZ administration. Both 1250mg/m2 (~105mg/kg) Sigma STZ as well as 150mg/kg Zanosar STZ resulted in the cessation of C-peptide production (i.e., to a level below the detection level of the respective assays). At 150mg/kg, we were able to successfully establish diabetes in nonhuman primates without residual beta cell function, and without associated morbidity. In contrast, high-dose Sigma STZ was associated with adverse effects, although the role of additional immunosuppressive therapy in the development of this damage remains uncertain.

Furthermore, we observed a triphasic response in blood glucose during the first 36h after STZ administration. Such a response has been described in rodents (63, 64) and monkeys

(62, 65). Interpretation of the triphasic response is that there is an initial decrease of insulin release as a result of inflammation and an inability of the beta cells to respond to glucose due to impaired glucose oxidation in the beta cell (63, 66); this explains the initial rise in blood glucose (phase I). Further destruction and disruption of the beta cells results in a massive release of insulin, leading to a fall in blood glucose level (phase II). Finally, there is a critical loss of beta cells (phase III), resulting in the diabetic state (64, 66). We documented a peak blood glucose after 8h (compared to 2-7h in the rat), followed by a steady fall until 17h (8-12h in the rat) and a subsequent rise with persistent hyperglycemia (>200mg/dL) after 30.3h (24h in the rat) (63, 64). Since a monkey that did not become successfully diabetic lacked this triphasic response, we suggest this triphasic response indicates the complete and successful induction of diabetes.

Reduction of early graft loss after intraportal pig-to-monkey islet transplantation

After establishing a model of diabetes in cynomolgus monkeys, we could begin to study pig-to-monkey islet transplantation in diabetic monkeys (**Chapter 8**). It has been well documented by several groups that large numbers of pig islets are rapidly lost after their intraportal transplantation into primate recipients (67-69, reviewed in 70), and we have confirmed this observation. In our study, the extent of the initial injury to the transplanted pig islets from IBMIR was largely estimated by the amount of dextrose required to prevent serious hypoglycemia, due to massive destruction of islets. A high dextrose requirement correlated with a short period of graft survival, as measured by blood glucose level, insulin requirement, and the level of porcine C-peptide. Complement depletion with cobra venom factor (CVF) or anticoagulation with dextran sulfate (DS), resulted in an important reduction of early graft loss, resulting in increased survival of the islet graft. Early graft survival was indicated by higher porcine C-peptide levels. However, even with complement depletion or anticoagulation, significant early graft loss could not be prevented completely.

It has been reported that DS alone results in both anticoagulation and inhibition of complement activity (69, 71). Our own observations suggest that, although effective as an anticoagulant, DS was markedly less effective in inhibiting complement activity. Other agents and approaches therefore require to be investigated to prevent IBMIR. For example, a combination of complement depletion (by CVF) and anticoagulation (by DS) may prove more successful.

The best result was obtained by our group when we doubled the number of islets transplanted to 80,000IEQ/kg in one monkey. Normoglycemia, without the need for exogenous insulin, and high porcine C-peptide levels were documented throughout the 5-day period of observation, indicating that the number of islets transplanted had compensated for the initial loss that resulted from IBMIR. It is perhaps important to note that the normal level of blood glucose in healthy monkeys is 50-70mg/dL. The monkey that was transplanted with 80,000IEQ/kg, was the only diabetic monkey in which this relatively low blood glucose levels,

or true normoglycemia, was obtained, and this was only achieved when porcine C-peptide levels were between 2.7-6.2ng/mL. In the other monkeys, graft function associated with lower porcine C-peptide levels (0.5-2.0ng/mL), which are actually high levels in healthy pigs, resulted in improvement of the diabetic status for up to two months, but not in normoglycemia. A potentially important observation is that C-peptide levels in healthy non-diabetic monkeys (>1.5ng/mL in our laboratory) are considerably higher than in healthy non-diabetic pigs (0.2-0.6ng/mL). Peculiar characteristics of monkey metabolism and/or of porcine islets may therefore be relevant to islet function in pig-to-nonhuman primate transplantation models.

As the number of porcine islets that could be made available for any single diabetic patient will be theoretically limitless, this observation may provide a simple way of overcoming the considerable barrier raised by IBMIR. However, to provide 80,000IEQ per kg for an 80kg patient might require a total of more than 6 million IEQ (requiring ~15-20 adult retired breeder pigs). On the other hand, the number may be significantly fewer since fasting C-peptide levels in healthy humans (1.18+/-0.06ng/mL (72)) are considerably lower than those in monkeys. Pig islet insulin production may therefore sustain normoglycemia more successfully in humans than in monkeys, necessitating the transplantation of significantly fewer porcine islets. Nevertheless, it would clearly be preferable to reduce the percentage loss of islets from IBMIR. Furthermore, although the elevation in liver enzymes after islet transplantation in the present study was only transient, there is the potential for portal vein thrombosis or liver injury after the intraportal transplantation of large numbers of islets.

In other studies, it has proved difficult to determine how much loss of islet function is a consequence of IBMIR and how much is a result of rejection. Increasingly, however, the loss from IBMIR is becoming more obvious (73, 74). Although medium-term survival of porcine islets in nonhuman primates has recently been reported in the apparent absence of specific treatment for IBMIR (1, 2), there is still a need to study and prevent IBMIR, since it would be ideal to obtain true normoglycemia with the smallest possible number of islets and with minimal immunosuppression. In our own studies using a costimulation blockade-based regimen, as originally introduced by Buhler et al (19), T cell-mediated acute cellular rejection does not appear to have been a major factor in graft loss, since there has been an absence of cellular infiltration in the liver and of a T cell-dependent elicited antibody response. It may be only when the problem of IBMIR has been resolved that low-dose immunosuppressive therapy will be able to be fully investigated.

GT-KO pigs have not been used previously as sources of islets for transplantation into primates. In our study, as anticipated, we did not find that adult GT-KO pig islets were less susceptible than WT pig islets to early destruction. It has been reported that carefully isolated adult WT islets do not express the Gal epitopes against which primate anti-pig antibodies are largely directed (9). Our own observations also indicate that endocrine islet cells, including beta cells, do not express Gal epitopes, although Gal-positive cells can be found in small

numbers, most likely as a consequence of imperfect enzymatic separation of the exocrine from the endocrine tissues or from the presence of vascular endothelial cells. In contrast, islets from GT-KO pigs show no staining for Gal. In isolated WT islets, the Gal-stained tissues do not include the insulin-producing beta cells.

However, early function between the GT-KO and WT islets cannot be strictly compared, as all WT islets were obtained from retired breeding sows whereas the GT-KO islets were not; it has been well-documented that islets from retired breeders have better post-transplantation characteristics (75, 76). The outcome may well have been different if neonatal or fetal islets (that express abundant Gal epitopes) had been transplanted. It has been shown in GT-KO mice that transplanted WT neonatal pig islets induce an increase in anti-Gal antibody titer, whereas GT-KO neonatal islets do not (10).

Summarizing our pig to monkey islet transplantation experiments: Early loss of pig islets occurred after intraportal transplantation in monkeys, even when attempts were made to inhibit IBMIR by CVF+heparin or DS administration. This early graft loss requires intensive further investigation. However, survival of viable islets up to two months with C-peptide levels that are high for pigs was documented. To date, there have been no reports on survival of porcine islets with C-peptide levels that would be physiologic for humans. Medium-term survival of islets with (for primates) low levels of C-peptide is currently feasible. It would be challenging to study functional pig islet survival in nonhuman primates, with C-peptide values that are in the normal range for the host. On the other hand, clinically acceptable survival of porcine islets may be closer than expected, since reported C-peptide levels for humans are lower than for monkeys (Chapter 2, Table 2). This would suggest that islet graft function with associated porcine C-peptide levels that would not be able to render a monkey normoglycemic, may be able to achieve normoglycemia in a human.

FUTURE PERSPECTIVES

Clinical islet xenotransplantation

Chapter 9 addresses the current position of clinical islet xenotransplantation. Although valuable data may be obtained from clinical trials of islet xenotransplantation, and although efforts towards clinical trials should not be unduly impeded, we have to question whether there is sufficient encouraging experimental data in nonhuman primate models to warrant further clinical studies at the present time. Over the past years, some data have become available from pig-to-nonhuman primate studies (77) but, except for two reports on successful survival of pig islets in cynomolgus monkeys for periods of several months using an immunosuppressive regimen that is unlikely to be clinically applicable (1, 2), there are no studies that support a conclusion that clinical trials are likely to be successful (77).

Although some patients have required less, or even no, insulin (at least temporarily) after pig islet transplantation, and thus the patients have individually benefited from clinical trials, it remains uncertain whether this was related to improved medical management, e.g., meticulous attention to diet, regular monitoring of blood glucose, excellent medical advice and management, etc., rather than to the function of the transplanted islets. Diabetic patients are likely to be more carefully monitored and more attentive to maintaining good control of their own condition when participating in a well-organized clinical trial.

The future may host more clinical islet xenotransplantation studies. If so, in the opinion of the IXA Ethics Committee, it would be mandatory that convincing experimental data in nonhuman primate models as to the efficacy of the approach are available to indicate the likelihood of a successful outcome and to justify exposure to potential risks. Careful monitoring (in collaboration with a recognized national authority) for potential transmission of infectious microorganisms is also surely mandatory. All aspects of the trial should be under the supervision of an institutional (and possibly a national) committee or authority. Such oversight and monitoring will surely not only safeguard the individual patient and the community, but will also increase the likelihood of obtaining valuable data from the trial, even if it is not fully successful in achieving its goals.

At this point in time, more experimental data from nonhuman primate models documenting convincing efficacy, focused on clinically-applicable immunosuppressive regimens, are needed to justify the initiation of clinical trials. These studies should also be designed to provide further data on the safety of the procedure, particularly with regard to the transfer of porcine viruses (including, but not limited to, PERV) to the nonhuman primate recipient of the porcine islets. A carefully-monitored clinical trial may then be justified even though the potential risk to the patients, and possibly for society, would not be zero.

The results required in a nonhuman primate model of porcine islet transplantation to justify progress to a clinical trial remain uncertain. The criteria for a clinical trial are particularly difficult to determine for patients with diabetes, since the disease is not as rapidly fatal as many other conditions for which xenotransplantation offers hope. A strong case could be made for a consensus meeting, and/or the setting up of an expert advisory committee (as convened by the International Society of Heart and Lung Transplantation in 2000 [78]) to determine these criteria.

Islet cell regeneration

Efforts have been made to find a successful treatment for type-1 diabetes, such as islet xenotransplantation. Despite progress in the field of transplantation, this has not yet resulted in a permanent solution. Rodent studies have given us hope for a new direction: regeneration of the patient's own beta cells, which was reviewed in **Chapter 10**. Preliminary studies in primates support anecdotal examples suggesting that beta cell regeneration might be

possible also in humans. If abrogation of autoimmunity can be safely achieved in a diabetic patient with an autotransplant of precursor cells transfected with HLA class II beta chain genes conferring resistance to the disease, while correcting his/her hyperglycemia using conventional insulin administration or an islet allotransplant, 'nature' will be left to heal the rest. It should also be possible to speed up the natural process of healing by endoscopic retrograde intraductal delivery of factors known to promote beta cell regeneration. Should this approach work satisfactorily, young diabetic patients will be permanently cured, without the need for long-term drug therapies associated with known troublesome consequences.

The highly interesting field of islet cell regeneration is one that should be fully investigated, particularly since it aims at a true 'cure' for diabetes, while successful islet transplantation can possibly only be considered a 'treatment'. However, at the moment there is insufficient evidence to support the assumption that this alternative to islet transplantation will lead to a cure for diabetes in the near future. Therefore, further research into islet xenotransplantation is fully justified, especially considering the progress that has been made over the past decade.

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Samenvatting en discussie

Het ultieme doel is het vinden van een definitieve behandeling van type 1 diabetes, waarmee de notoire lange-termijn complicaties voorkomen kunnen worden, alsmede de beperkingen die insulinebehandeling geeft voor het dagelijks leven. Alvleeskliertransplantatie en transplantatie van eilandjes van Langerhans van een humane donor zijn potentiële oplossingen, alhoewel deze beperkt worden door onder andere chirurgische complicaties en het tekort aan donororganen.

Een mogelijke oplossing voor het tekort aan donororganen kan worden gevonden in xenotransplantatie, waarbij het varken gebruikt wordt als bron van eilandjes van Langerhans. Insuline van varkens werd immers decennia lang succesvol gebruikt voor de behandeling van diabetespatiënten. Helaas bestaat het probleem dat organen en weefsel van gewone, 'wild-type' (WT) varkens snel worden afgestoten na xenotransplantatie. Bij deze afstotingsreactie spelen antilichamen in het serum van de ontvanger, gericht tegen het varkenstransplantaat, een belangrijke rol. Eerder is ontdekt dat zogenaamde anti-Gal antilichamen, gericht tegen het 'Gal-epitoop' op cellen van varkens, een centrale rol vervullen in de afstoting direct na transplantatie van een varkenstransplantaat. Er zijn varkens ontwikkeld die homozygoot knock-out zijn voor het α1,3-galactosyltransferase gen; de galactosyltransferase-knock out (GT-KO) varkens. Hiermee zijn nieuwe mogelijkheden gecreëerd voor varken naar primaat xenotransplantatie, aangezien deze varkens geen Gal-epitoop op hun cellen tot expressie brengen. Klinische trials kunnen niet beginnen voordat resultaten van knaagdier studies gevalideerd zijn in een pre-klinisch model. Dit legitimeert studies in niet-humane primaten.

Deze dissertatie begint met een review van de voorgaande ervaring met varken-naar-niet-humane primaat eilandjestransplantatie (**Hoofdstuk 2**). De soort varkenseilandjes wordt besproken, te weten eilandjes van foetale, neonatale en volwassen varkens. Het lijkt dat eilandjes van volwassen varkens de beste eigenschappen bezitten, zowel vanuit logistiek als immunologisch oogpunt, om diabetes te behandelen in primaten. Met de huidige isolatietechnieken kunnen grote hoeveelheden volwassen eilandjes van goede kwaliteit worden verkregen.

Verder worden modellen om diabetes te induceren in niet-humane primaten beschreven. Een bruikbaar experimenteel model voor inductie van diabetes in niet-humane primaten, is gevonden in de toediening van het voor insuline producerende cellen toxische streptozotocin (STZ). Het is echter onduidelijk of STZ volkomen irreversibel diabetes induceert.
Verlenging van de overleving van getransplanteerde varkenseilandjes in niet-humane primaten gaat helaas langzaam. Met de vele beschikbare immunosuppressiva zal de uitdaging liggen in het bepalen van de optimale combinatie. In het bijzonder bij een ziekte zoals diabetes, welke niet snel fataal hoeft te zijn, bestaat de noodzaak om klinisch toepasbare protocollen van immunosuppressiva te ontwikkelen die niet geassocieerd zijn met potentieel levensbedreigende bijwerkingen.

ANTI-NONGAL ANTILICHAMEN

Transplantatie van een orgaan van een gewoon, 'wild-type' (WT) varken in een immuungesuppresseerde primaat resulteert gewoonlijk binnen enkele minuten tot uren in hyperacute afstoting van het orgaan. De rol die anti-Gal antilichamen hierbij hebben, is bekend. De rol van andere antilichamen in primaten gericht tegen varkensorganen en/of weefsel, zogenaamde anti-nonGal antilichamen, hebben we kunnen onderzoeken nadat de GT-KO varkens beschikbaar kwamen.

Anti-nonGal antilichamen bij bavianen

In **Hoofdstuk 3** beschrijven we het voorkomen van deze anti-nonGal antilichamen in het serum van bavianen, en we onderzochten of deze antilichamen in staat waren bepaalde varkenscellen (Peripheral Blood Mononuclear Cells, PBMC) te beschadigen, door cytotoxiciteit van het serum te testen tegen PBMC van GT-KO varkens. Deze resultaten hebben we vergeleken met de reactiviteit van anti-varkens antilichamen tegen PBMC van WT varkens.

We toonden aan dat vrijwel alle bavianen antilichamen bezaten tegen WT varkenscellen, maar slechts de helft bezat deze antilichamen tegen GT-KO varkenscellen. Ook wordt beschreven dat deze anti-nonGal antilichamen significant minder cytotoxisch zijn dan de anti-Gal antilichamen. Desalniettemin initiëren anti-nonGal antilichamen in de laboratoriumsetting wel degelijk een cytotoxische reactie. Deze reactie zou overleving van een GT-KO transplantaat in vivo kunnen ondermijnen.

Verder wordt beschreven dat er bij bavianen na transplantatie van GT-KO organen een anti-nonGal antilichaam reactie op gang komt. Deze opgewekte anti-nonGal antilichamen zijn ook cytotoxisch. Dit illustreert dat anti-nonGal antilichamen ook een rol hebben in de afstoting van varkensorganen, alhoewel veel minder krachtig dan de anti-Gal antilichamen.

Anti-nonGal antilichamen bij mensen

In **Hoofdstuk 4** rapporteren we over de incidentie van anti-nonGal antilichamen in het serum van mensen en over de cytotoxiciteit van deze antilichamen met betrekking tot varkenscellen (PBMC). Alle geteste sera bevatten anti-Gal antilichamen, tegenover twee-derde van de sera die anti-nonGal antilichamen bevatten. Net als bij bavianen was de cytotoxiciteit van deze anti-nonGal antilichamen significant lager dan de cytotoxiciteit van de totale anti-varkens antilichamen. Dit betekent dat een GT-KO transplantaat weliswaar geschikter is dan een WT transplantaat, maar dat anti-nonGal antilichamen nog steeds een probleem vormen en dat zij de overleving van het transplantaat kunnen bedreigen.

Antilichamen kunnen bij mensen gedurende het gehele leven gevormd worden, bijvoorbeeld door expositie aan virussen of bacteriën. Zo zijn patiënten die op een wachtlijst staan voor

transplantatie onder te verdelen in patiënten met een lage, en patiënten met een hoge reactiviteit van hun serum tegen humane donoren. Patiënten met een hoge reactiviteit (zogenaamd allogesensitiseerd) hebben meer moeite een geschikt donororgaan te vinden, vanwege de reactiviteit van hun serum tegen het donororgaan. Wij hebben onderzocht of patiënten met een hoge reactiviteit ook een hogere reactiviteit tegen xenotransplantaten hebben dan niet gesensitiseerde patiënten. Onze resultaten laten zien dat dit niet het geval is. Dit betekent dat deze patiënten, die een kleine kans hebben een geschikt humaan donororgaan te vinden, mogelijk als eerste in aanmerking komen voor een xenotransplantaat.

Het is van belang om te onderzoeken waar de nonGal-antilichamen precies uit bestaan. Tot op heden is er echter pas één antilichaam gedefinieerd dat als anti-nonGal antilichaam bij mensen (maar niet bij bavianen of cynomolgus apen) aangemerkt kan worden; het Hanganutziu-Deicher antilichaam. Er ligt een belangrijke rol voor toekomstig onderzoek om andere anti-nonGal antilichamen te identificeren. Ook zou het interessant zijn om te onderzoeken of de anti-nonGal antilichamen uit het serum van de ontvanger weggevangen kunnen worden door middel van immunoabsorptie, waarbij de anti-nonGal antilichamen geabsorbeerd worden uit het serum. Een andere potentiële mogelijkheid om afstoting door anti-nonGal antilichamen te voorkomen is door te transplanteren in het eerste levensjaar, voordat deze antilichamen gevormd zijn. Dit wordt in hoofdstuk 6 beschreven.

Anti-nonGal antilichamen bij cynomolgus apen

Hoofdstuk 5 beschrijft de aanwezigheid van anti-nonGal antilichamen in het serum van cynomolgus apen en vergelijkt de niveaus van antilichamen tussen cynomolgus apen, bavianen en mensen. We vonden wederom een significant lager niveau van anti-nonGal antilichamen dan van anti-varkens antilichamen. We toonden echter ook aan dat driekwart van de geteste sera anti-nonGal antilichamen bevatten die cytotoxisch zijn, alhoewel het niveau van cytotoxiciteit van anti-nonGal antilichamen lager is dan van anti-varkens antilichamen.

Bij cynomolgus apen is het niveau van anti-nonGal antilichamen en de cytotoxiciteit van deze antilichamen significant hoger dan bij bavianen en mensen. Dit zou betekenen dat de baviaan wat betreft anti-nonGal antilichamen dichter bij de mens staat dan de cynomolgus aap, en hierdoor geschikter zou kunnen zijn als preklinisch model.

Anti-nonGal antilichamen bij kinderen

Natuurlijke anti-varkens antilichamen ontstaan gedurende de eerste maanden van het bestaan. We hebben beschreven in **Hoofdstuk 6** dat de ontwikkeling van (cytotoxische) anti-nonGal antilichamen bij pasgeborenen heel langzaam op gang komt en dat er geen correlatie bestaat tussen het niveau van deze antilichamen en de leeftijd gedurende het eerste levensjaar. Daarentegen worden cytotoxische anti-varkens antilichamen vroeg in het eerste levensjaar gevormd en het niveau stijgt gedurende dit levensjaar. De ontwikkeling

van anti-varkens antilichamen is evenredig met de ontwikkeling van anti-bloedgroep antilichamen. In onze studie vonden we dat deze antilichamen bij bavianen op de leeftijd van ongeveer vier maanden gevormd zijn.

Dit biedt perspectieven voor eventuele toekomstige transplantatie van GT-KO weefsel en/of organen bij pasgeborenen, aangezien zij nog geen antilichamen bezitten die antilichaam-gemedieerde afstoting kunnen veroorzaken. Dit principe is al beschreven voor harttransplantatie bij pasgeborenen, waarbij gebruik gemaakt is van een hart van een donor met een andere bloedgroep. Bij pasgeborenen werden anti-bloedgroep antilichamen niet meer gevormd, wanneer het bloedgroep-incompatibele hart getransplanteerd werd voordat anti-bloedgroep antilichamen zich ontwikkeld hebben. Dit zou voor anti-nonGal antilichamen ook het geval kunnen zijn, waardoor de transplantatie kan plaatsvinden in afwezigheid van anti-nonGal antilichamen. Wel dient de T-cel gemedieerde afstoting gesupprimeerd te worden, zoals bij klinische harttransplantaties.

TRANSPLANTATIE VAN VARKENSEILANDJES VAN LANGERHANS IN APEN

Ons doel was te bestuderen hoe diabetische apen genezen kunnen worden door transplantatie van varkenseilandjes. Om optimaal de functie van het eilandjestransplantaat te bestuderen, is het van belang om deze te transplanteren in volledig diabetische apen.

Inductie van diabetes

In **Hoofdstuk 7** beschrijven we een model om op een veilige manier diabetes te induceren in cynomolgus apen. Dit is de eerste studie over veilige inductie van diabetes in niet-humane primaten door middel van een hoge dosis, voor insulineproducerende cellen toxische, Zanosar streptozotocin (STZ). Zanosar STZ wordt gebruikt bij patiënten met gemetastaseerde eilandjes tumoren en lijkt minder bijwerkingen te hebben dan het veelgebruikte Sigma STZ. Wellicht wordt dit veroorzaakt door grotere zuiverheid en minder variabiliteit. Na de behandeling van de apen met een hoge dosis Zanosar STZ was er geen C-peptide meer detecteerbaar in het serum van de apen. C-peptide is een afsplitsingsproduct van het endogeen geproduceerde insuline en daarmee een maat voor de hoeveelheid geproduceerd insuline.

Er is veel discussie geweest over de optimale dosis van STZ om bij niet-humane primaten diabetes te induceren. Lage doses zijn veelal onvoldoende gebleken om de beta-cellen van de pancreas volledig uit te schakelen. Hoge doses zijn echter geassocieerd met nier- en levertoxiciteit. Vandaar dat het van groot belang is om een optimale dosis te vinden waarbij volledige diabetes en een minimaal risico op bijwerkingen gewaarborgd worden. Wij hebben aangetoond dat diabetes consistent kan worden geïnduceerd met een hoge dosis STZ van zowel het Zanosar als het Sigma type. Alleen Zanosar STZ bleek dit te bewerkstelligen met weinig tot geen morbiditeit.

Verder hebben we beschreven dat er bij de inductie van complete diabetes een trifasische bloedglucose respons optreedt in de eerste 36 uur. Hierbij stijgt in de eerste fase de bloedglucose door een afname van insulineproductie door de beta-cellen. In de tweede fase treedt massale destructie van de beta-cellen op wat resulteert in een sterke afname van de bloedglucose waarde. In de derde fase stijgt de bloedglucose weer, wat ditmaal betekent dat de definitieve diabetische staat is bereikt. Afwezigheid van deze respons betekent onvolledige inductie van diabetes.

Reductie van het verlies van eilandjes na intraportale transplantatie van varkenseilandjes in apen

Na het ontwikkelen van een model voor inductie van diabetes in cynomolgus apen, konden we beginnen met de varken-naar-aap eilandjestransplantatie in diabetische apen (**Hoofdstuk 8**). Wij, maar ook andere onderzoeksgroepen, hebben beschreven dat grote hoeveelheden eilandjes verloren gaan in de eerste periode na transplantatie van de eilandjes in de poortader. Deze reactie wordt de 'instant blood-mediated inflammatory reaction' (IBMIR) genoemd. Stolling, complement activatie en bloedplaatjes activatie spelen een belangrijke rol in de destructie van de eilandjes. Wij hebben een tweetal methoden geïntroduceerd en/of getest die deze reactie tegengaan. Complement depletie in de apen door middel van cobra venom factor (CVF) en antistolling met behulp van dextran sulfate (DS) heeft geresulteerd in een belangrijke afname van het verlies van de eilandjes kort na transplantatie. Dit heeft geleid tot (tijdelijke) insuline onafhankelijkheid, langdurig verminderde insulinebehoefte, betere controle van bloedglucosewaarden en langdurig functioneren van de eilandjes. Echter, zelfs met complementdepletie of anticoagulatie, kon de destructie van eilandjes niet volledig voorkomen worden. Er zal in toekomstige studies gezocht moeten worden naar andere methoden om afbraak van eilandjes te voorkomen.

Varkens, apen en mensen hebben verschillende normaalwaarden voor serum C-peptide niveaus (Hoofdstuk 2, tabel 2). We hebben overleving van varkenseilandjes in apen gezien met bijbehorende C-peptide niveaus die hoog zijn in vergelijking met C-peptide niveaus in varkens. Momenteel zijn er geen studies bekend waarbij overleving van eilandjes gerapporteerd is met C-peptide niveaus die fysiologisch zouden zijn voor de mens. Middellange termijn overleving van eilandjes met (voor primaten) lage niveaus van C-peptide is momenteel mogelijk. Het zou echter een uitdaging zijn om overleving van functionele varkenseilandjes te bestuderen in niet-humane primaten, met C-peptide waarden die in de normale range voor de ontvanger liggen. Klinisch acceptabele overleving van varkenseilandjes kan echter dichterbij zijn dan verwacht, aangezien C-peptide waarden voor mensen lager zijn dan voor apen. De situatie zou kunnen bestaan dat de insulineproductie van getransplanteerde eilandjes (met bijbehorende C-peptide niveaus) onvoldoende is om een aap normoglycemisch te maken, terwijl diezelfde insulineproductie wel voldoende is om normoglycemie bij de mens te bewerkstelligen.

Het is moeilijk gebleken om te bepalen hoeveel eilandjes verloren gaan als gevolg van IBMIR en hoeveel er verloren gaan als gevolg van afstoting. Toenemend wordt het verlies door IBMIR beschreven als belangrijke factor. Alhoewel middellange termijn overleving van varkenseilandjes in niet-humane primaten onlangs beschreven is zonder dat hierbij specifieke therapie gericht op IBMIR gegeven werd, bestaat nog steeds de noodzaak om IBMIR te bestuderen en te voorkomen. Het uiteindelijke doel is namelijk volledige normoglycemie te behalen met de kleinst mogelijke hoeveelheid eilandjes en met minimale immunosuppressiva. In onze eigen studies hebben we gebruik gemaakt van een zogenoemd 'co-stimulatie blokkade' immunosuppressiva protocol. Hierbij hadden we geen aanwijzingen voor een rol van T-cel gemedieerde acute afstoting, aangezien er geen cellulaire infiltraten in de lever zijn gevonden en er ook geen T-cel gemedieerde antilichaamreactie opgewekt werd bij de ontvanger. Wellicht dat de ontwikkeling van een lage-dosis immunosuppressiva protocol pas plaats kan hebben wanneer het probleem van IBMIR opgelost is.

GT-KO varkens zijn niet eerder bestudeerd als donoren van eilandjes voor transplantatie in primaten. Zoals verwacht, vonden wij in onze studie niet dat volwassen GT-KO eilandjes minder gevoelig waren voor vroege afbraak na transplantatie dan WT eilandjes. Het is bekend dat zorgvuldig geïsoleerde volwassen WT eilandjes geen Gal tot expressie brengen, het voor primaten voornaamste antigeen waartegen antilichamen bestaan. Onze eigen observaties indiceren ook dat endocriene eilandjescellen, inclusief beta-cellen, geen Gal-epitopen tot expressie brengen. Wanneer er foetale of neonatale eilandjes gebruikt zouden zijn (waarbij de WT eilandjes wel Gal tot expressie brengen), zou er naar verwachting wel verschil geconstateerd zijn.

TOEKOMSTPERSPECTIEVEN

Klinische transplantatie van eilandjes van Langerhans

In **Hoofdstuk 9** wordt de huidige positie van klinische eilandjes xenotransplantatie besproken. Hoewel er waardevolle data verkregen worden uit klinische studies, en hoewel stappen in de richting van klinische trials volledig doorgang moeten hebben, moeten we ons afvragen of er op dit moment voldoende experimentele data in niet-humane primaat modellen zijn om verdere klinische studies te rechtvaardigen.

Klinische studies uit het verleden hebben laten zien dat sommige patiënten (tijdelijk) minder, of zelfs geen insulinetherapie nodig hadden na transplantatie van varkenseilandjes. Deze patiënten hebben dus individueel voordeel gehad van klinische trials, alhoewel het onduidelijk blijft of dit gerelateerd was aan verbeterde medische behandeling, zoals bijvoorbeeld strikte aandacht voor het dieet, strikte bloedglucose metingen, goede medische zorg en advies.

De laatste jaren zijn gegevens beschikbaar gekomen uit varken-naar-niet-humane primaat

eilandjestransplantatie studies. Met uitzondering van twee studies die rapporteren over de succesvolle overleving van varkenseilandjes voor perioden van enkele maanden, gebruik makend van immunosuppressiva die zeer waarschijnlijk klinisch niet toepasbaar zijn, zijn er momenteel geen studies die erop wijzen dat klinische trials kans van slagen hebben.

In de toekomst zouden echter nieuwe klinische trials kunnen plaatsvinden. In dat geval is de Ethische Commissie van de International Xenotransplantation Association (IXA) van mening dat deze gebaseerd dienen te zijn op overtuigende experimentele data uit niet-humane primaat onderzoeken. Hierin moeten zowel effectiviteit en waarschijnlijkheid van een succesvolle uitkomst onderzocht zijn, alsook de potentiële risico's. Zorgvuldige observatie, in samenwerking met een erkende nationale autoriteit, voor mogelijke transmissie van infectieuze micro-organismen is een absoluut vereiste. Alle aspecten van de trial dienen onder supervisie van een institutionele (en mogelijk een nationale) commissie of autoriteit plaats te vinden. Deze supervisie en monitoring zullen niet alleen de individuele patiënt en de maatschappij beschermen, maar zullen ook de kans op het verkrijgen van waardevolle data vergroten, zelfs wanneer de trial niet succesvol zou zijn.

Welke resultaten in een niet-humane primaat-studie vereist zijn, alvorens klinische studies te kunnen starten, blijft onduidelijk. De criteria voor klinische eilandjestransplantatie trials zijn bijzonder moeilijk te definiëren voor diabetespatiënten, aangezien de ziekte niet zo snel fataal hoeft te zijn als vele andere ziekten waarvoor xenotransplantatie hoop biedt. Er zou gestreefd moeten worden naar een consensus bijeenkomst, en/of het oprichten van een adviescomité van experts om deze criteria te bepalen.

Eilandjesregeneratie

Er zijn inspanningen gedaan om een succesvolle behandeling van type 1 diabetes te vinden, zoals xenotransplantatie van eilandjes van Langerhans. Ondanks de progressie op het gebied van transplantatie heeft dit nog niet geresulteerd in een permanente oplossing. Knaagdierstudies hebben ons hoop gegeven op een nieuwe richting: regeneratie van de eigen beta-cellen van de patiënt, waarvan **Hoofdstuk 10** een review bevat. Pilot studies in primaten ondersteunen anekdotische voorbeelden dat beta-cel regeneratie mogelijk zou kunnen zijn bij mensen. De auto-immuniteit van diabetespatiënten zou veilig een halt toegeroepen kunnen worden door bijvoorbeeld eigen eilandjes te transplanteren, waarbij genen ingebracht zijn die resistentie tegen de auto-immuniteit teweegbrengen in HLA klasse II beta ketens genen. De 'natuur' kan dan zelf de diabetes genezen. Het zou ook mogelijk zijn om dit proces van natuurlijke genezing te versnellen door endoscopisch retrograad in de ductus pancreaticus factoren in te brengen die de beta-cel regeneratie bespoedigen. Mocht deze benadering werken, dan zouden nieuwe diabetespatiënten voorgoed te genezen zijn, zonder de noodzaak voor levenslange insulinetherapie en de lange termijn complicaties van de ziekte.

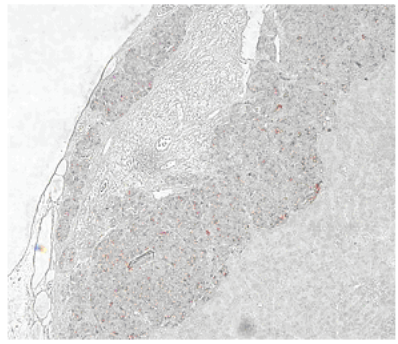
Deze zeer interessante richting van eilandjesregeneratie dient onderzocht te worden, in het bijzonder omdat het een echte *genezing* van diabetes zou betekenen, terwijl succesvolle eilandjestransplantatie zou kunnen leiden tot permanente *behandeling* van de ziekte. Echter, verder onderzoek op het gebied van xenotransplantatie van eilandjes van Langerhans is volledig gerechtvaardigd, in het bijzonder gezien de progressie die de afgelopen jaren gemaakt is in preklinische studies.

Appendices

COLOR FIGURES

Chapter 2, Figure 1:
(A) Fetal pig ICC, transplanted under the kidney capsule of a mouse, stained with anti-insulin antibodies, **(B)** Neonatal pig ICC, after isolation, stained in vitro with dithizone (dithizone stains red for insulin-containing cells), **(C)** Adult pig islets, after purification, stained in vitro with dithizone.

A.



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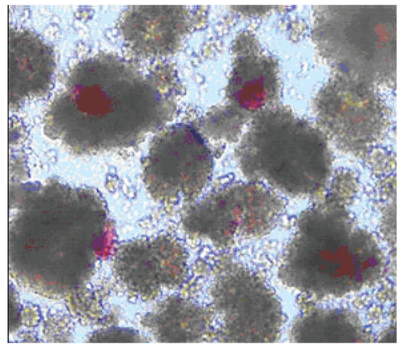
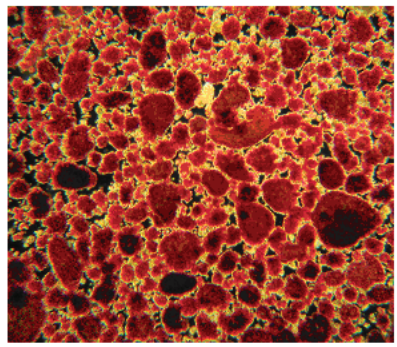
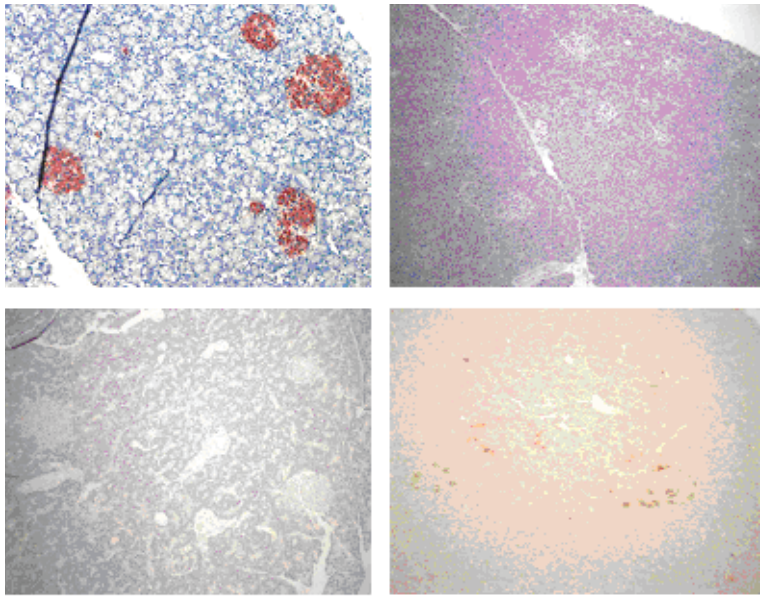
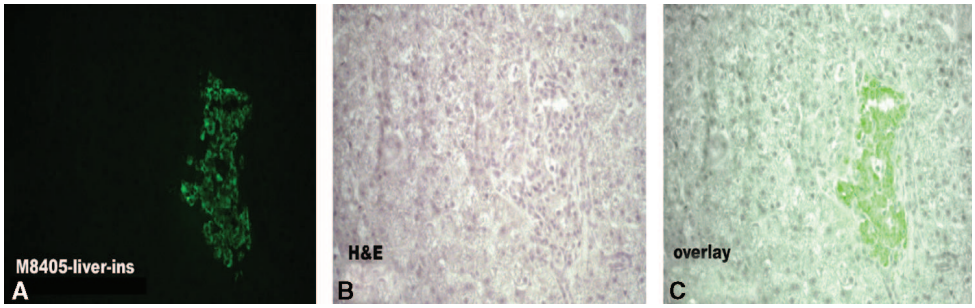


C.

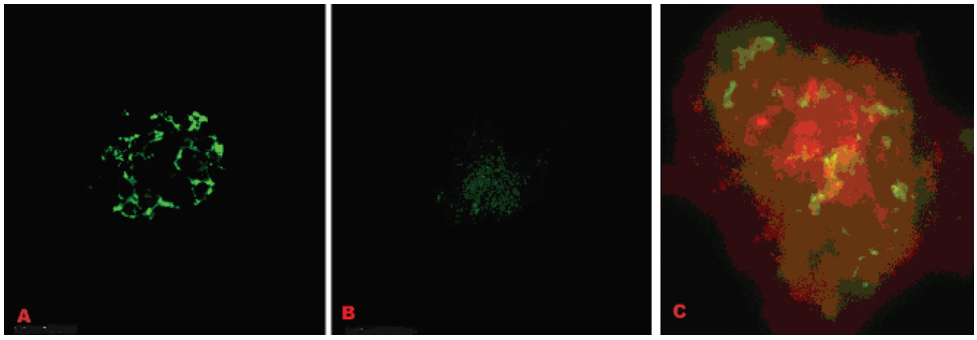




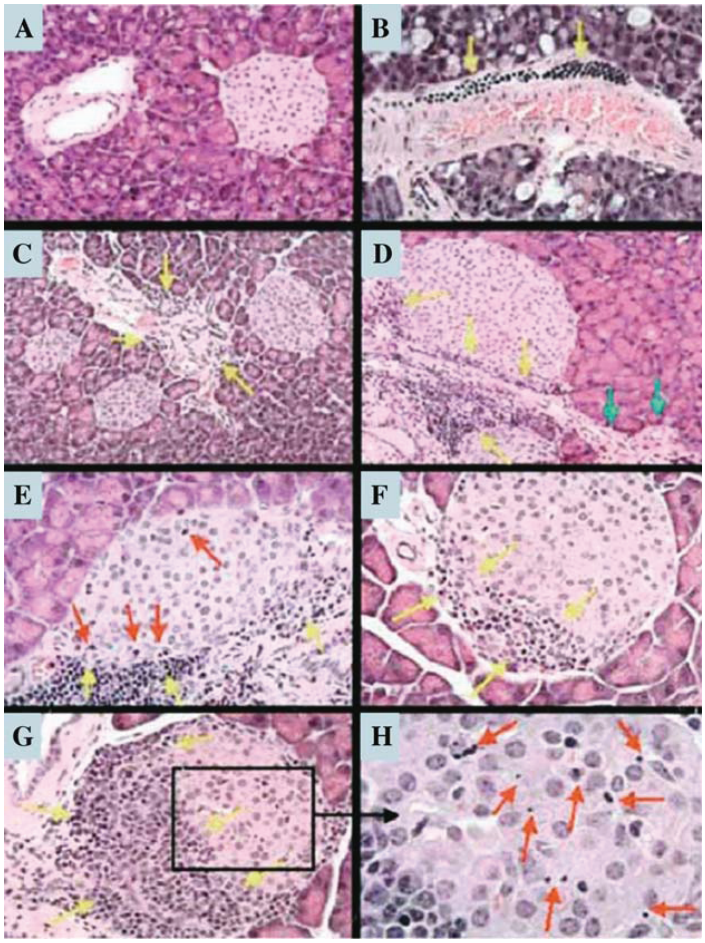
Chapter 7, Figure 4:
Insulin-positive cells in pancreatic tissue of normal monkey **(A)** and after 150mg/kg Zanosar STZ **(B)**, 1250mg/m2 (~105mg/kg) Sigma STZ **(C)**, and 60mg/kg Sigma STZ **(D)**. No insulin-positive cells are seen after high-dose STZ **(B, C)**; there are still insulin-positive cells present after low-dose STZ **(D)**.



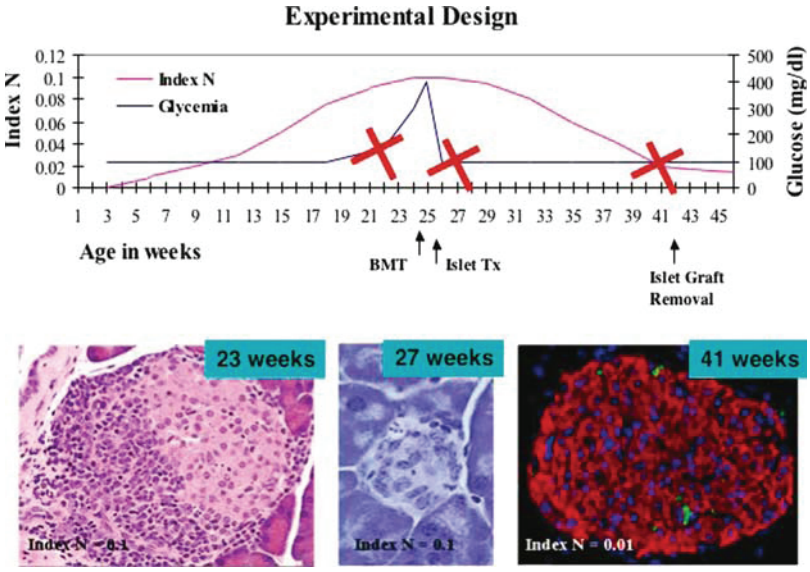
Chapter 8, Figure 4:
Microscopic section of the liver in a monkey (M8405) 7 days after the Tx of 80,000IEQ/kg, demonstrating islet grafted tissue stained for insulin (green fluorescence indicates insulin; magnification x20).



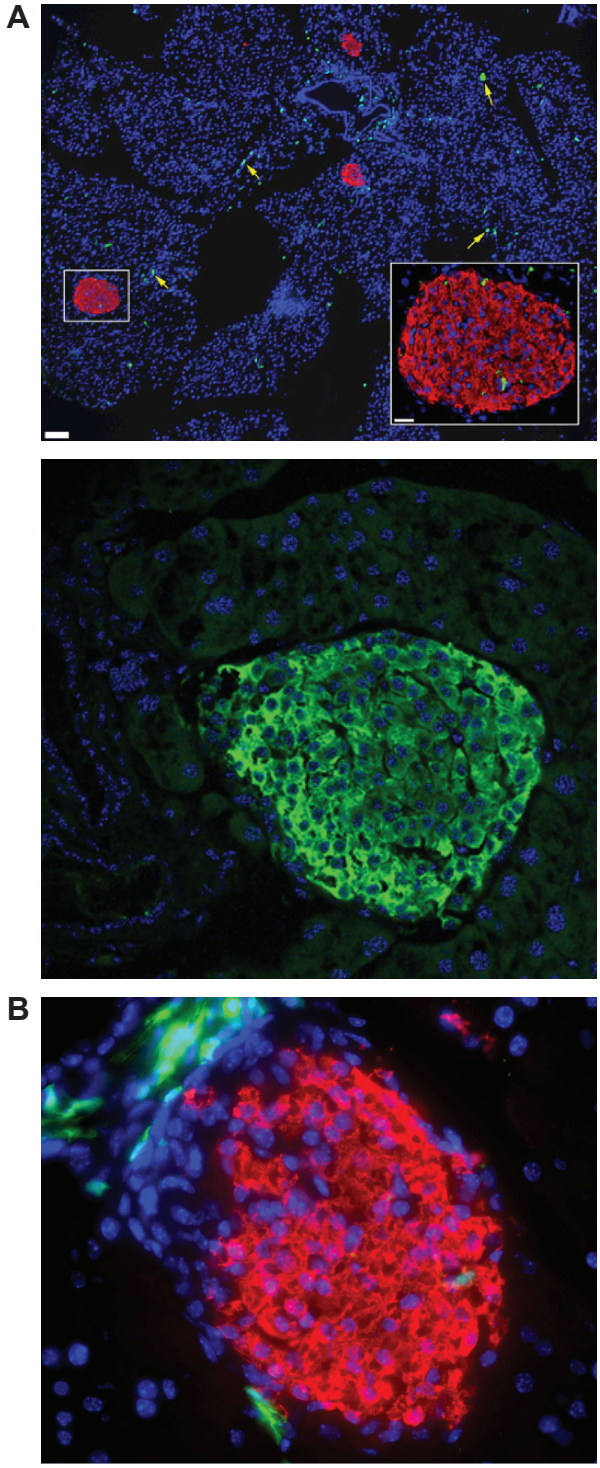
Chapter 8, Figure 6:
Isolated islets from **(A)** WT and **(B)** GT-KO pigs, stained with fluorescein-labeled Griffonia simplicifolia 1-isolectin B4 (green), a marker for Gal (magnification x20).
(C) Isolated WT pig islets immunostained for insulin (red) and fluorescein-labeled Griffonia simplicifolia 1-isolectin B4 (green), demonstrating that the insulin-producing beta cells do not stain for Gal (magnification x40).



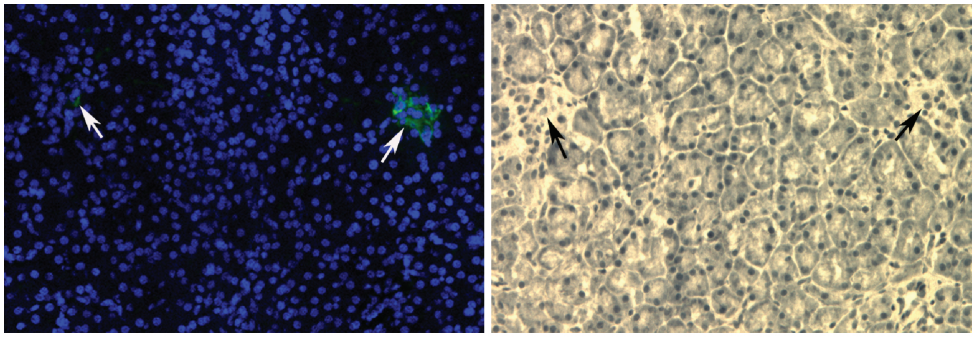
Chapter 10, Figure 3:
Scoring of the different stages of destruction of islets of Langerhans during diabetogenesis. Specimens of pancreata from NOD mice of different age were stained with H&E. Magnification for A to G x400, for H x1000. **(A)** Score 0: Normal pancreatic tissue. Neither morphological abnormalities nor mononuclear cell (MNC) infiltration or retention in the pancreatic vessels are present. **(B)** Score 1: MNC vascular retention (yellow arrows). No evident pathological features in pancreatic morphology. **(C)** Score 2: MNC perivascular infiltration (yellow arrows) of the vessels adjacent to the islets; islets maintain a normal morphology. **(D)** Score 3: MNC infiltration in the periphery of the islets (yellow arrows) and in the perivascular area of the adjacent vessels (compare to intact area distant from the islets, green arrows). **(E)** Score 4: The insulitis in the periphery of islets (yellow arrows) is associated with apoptosis (red arrows). **(F)** Score 5: The infiltration of islets by MNC (yellow arrows) is advanced, but not exceeding one third of islet section. **(G)** Score 6: More than one third of the endocrine tissue of the islet is infiltrated by the MNC (yellow arrows). This stage of insulitis is consistently concomitant with extensive apoptosis, presumably of both endocrine and infiltrating cells **(H:** red arrows**)** (60).



Chapter 10, Figure 5:
Schematic representation of the protocol used to test regeneration (or rescue) of the beta cell in diabetic NOD mice.
In NOD mice, the infiltration of autoreactive T cells into the islets of Langerhans (resulting in insulitis) begins at around 4 weeks of age. At 20 to 23 weeks, ~85% of female mice are diabetic, i.e., their glycemia is > 300 mg/dl. When successfully transplanted with bone marrow (BMT) from a non-diabetes-prone donor and hematopoietic chimerism is established, the NOD mouse no longer shows signs of autoimmune activity. However, while there is no more evidence of insulitis in the endogenous pancreas, there is also no sign of insulin production (no red staining). Three to four months after BMT, new insulin-positive cells (shown in red) are present throughout the endogenous pancreas. Thus, when the islets transplanted under the kidney capsule (to maintain euglycemia until regeneration takes place), are removed by nephrectomy, the mice remain non-diabetic (61). For "Index N" morphometric scoring system see ref. 60.



Chapter 10, Figure 6:
Regeneration (or rescue) of the endogenous pancreas in a diabetic NOD mouse after obliteration of the autoimmune process via allogeneic BMT.
(A) The regenerated endocrine tissue of a chimeric NOD mouse becomes evident after ~4 months from the BM transplant and takes the shape of cell agglomerates that resemble but are not identical to islets of a non-diabetic animal. Insulin is in red (61). **(B)** Comparison between an islet of Langerhans of a non-diabetic B6 mouse **(A)** with insulin stained green, and a newly formed insulin producing cell agglomerate (in red) in the pancreas of a diabetic NOD mouse treated with BM cells from a non-diabetes-prone, B6-GFP-transgenic donor **(B)**. It is possible to observe that the latter does not have the well organized cell structure of a normal islet and that the majority of the transplanted BM cells (in green) do not directly participate in the regeneration of the endogenous pancreas: there are no double-positive (orange) cells in the newly-formed islets. The donor cells appear to be located close to possibly existing juxta-ductal precursor cells, which may be activated by BM cell–secreted factors (45).



Chapter 10, Figure 7:
Newly formed insulin-producing cells in the diabetic monkey.
After 3 to 4 months from STZ injection and diabetes induction, insulin-producing cells are appearing in the monkey endogenous pancreas, eventually forming islet-like conglomerates of insulin-positive cells indicated by the arrows. Immunofluorescence on the left (insulin in green) and H&E on the right, of two consecutive tissue sections (Magnification 20x).

LIST OF ABBREVIATIONS

AAV	adeno-associated-virus
ACT	activated clotting time
AHXR	acute humoral xenograft rejection
APC	antigen presenting cell(s)
aPTT	activated partial thromboplastin time
AS-ODN	antisense oligodeoxynucleotides
ATG	anti-thymocyte globulin
BM	bone marrow
BMT	bone marrow transplantation
CDC	complement-dependent cytotoxicity
CVF	cobra venom factor
DC	dendritic cell(s)
DKO	double knockout
DM	diabetes mellitus
DS	dextran sulfate
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
EMT	epithelial-to-mesenchymal
Gal	galactoseα1,3galactose epitope
GFP	green fluorescent protein
GLP	glucagon-like peptide
GnT III	N-acetylglucosaminyltransferase-III
GT-KO	α1,3-galactosyltransferase gene-knockout
GVHD	graft versus host disease
HAR	hyperacute rejection
HD	Hanganutziu-Deicher antigen
hDAF	human decay-accelerating factor
hIPC	human islet precursor cells
HLA	human leucocyte antigen
HSC	hematopoietic stem cells
IBMIR	instant blood-mediated inflammatory reaction
ICA	islet cell antibodies
ICC	islet cell clusters
IEQ	islet equivalents
IFN	interferon
IGF	insulin-ike growth factor
iv	intravenous
ivGTT	intravenous glucose tolerance test
LIF	leukemia inhibitory factor

MET	mesenchymal-to-epithelial transition
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MNC	mononuclear cell(s)
NF	nuclear factor
NOD	nonobese diabetic
NS	not significantly different
PBMC	peripheral blood mononuclear cells
PRA	panel reactive antibodies
SLA	swine leukocyte antigens
STZ	streptozotocin
T1D	type 1 diabetes
TBI	total body irradiation
TCR	T cell receptor(s)
Tx	transplantation
WT	wild-type

CURRICULUM VITAE

Pleunie Rood was born on May 19th, 1978 in Delft, The Netherlands. After graduating *cum laude* from high school at the Sint Maartens College in Voorburg in 1996, she studied medicine at Maastricht University and at Erasmus University Rotterdam.
During medical school, she worked for two years as a student-assistant at the department of Anatomy and Embryology, Maastricht University (Prof.dr. J. Drukker). She performed several electives at universities and hospitals abroad. In 1998 she followed an elective in perinatology, at the University of Ljubljana, Slovenia. The research elective 'Diagnosing diabetes in a vascular unit' was performed in 2000 at the University of Pretoria, South Africa (Prof.dr. P. Rheeder). In 2003 she conducted a combined clinical and research elective in tropical medicine in Rubya, Tanzania (Dr. Overbosch, Harbour Hospital, Rotterdam). In 2003, she graduated from medical school *cum laude*.
She started her medical career as a surgical intern at the Reinier de Graaf Gasthuis, Delft (Dr. L.P.S. Stassen and Dr. M. van der Elst).
In April 2004, she moved to Pittsburgh, PA, USA for a two-year research fellowship in transplant surgery at the Thomas E. Starzl Transplantation Institute, University of Pittsburgh (Prof.dr. D.K.C. Cooper), and the Division of Immunogenetics, Children's Hospital of Pittsburgh (Prof.dr. M. Trucco). The studies performed during this fellowship form the basis for this thesis.
In July 2006, she started her residency in Emergency Medicine at Erasmus MC in Rotterdam, The Netherlands (Prof.dr. P. Patka).

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