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Expert Review

Facilitating Physiologic Self-Regeneration: A Step Beyond Islet Cell Replacement

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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, whereas 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 reestablishment 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.

KEY WORDS: adult stem cells; autoimmunity; beta cell regeneration; islet transplantation; type 1 diabetes.

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 nonbeta 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, which offer more precise management of glucohomeostasis, diabetics must monitor their blood glucose levels several times a day 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 lifestyle, 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,

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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, lifelong 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 transplantation, it has been reported that recipients experience benefits in terms of life expectancy (10). Pancreas transplantation alone 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 because of 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 5 years ago when the Edmonton group reinvigorated this field by reporting their experience treating seven patients consecutively who became insulin-independent (18). Insulin independence 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 50–80% 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 (22–24). 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 2–5 ml. 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, 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 allorejection 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 the 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 all eight 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 of posttransplantation (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 to realize the goal of curing T1D. For example, regeneration of endocrine pancreas function has been documented after partial pancreatectomy, and in streptozotocin (STZ)-treated animal models, including mice (39) and rats (40–42), and there are sporadic reports involving spontaneous remission in T1D patients (15,43), as well as evidence for islet neogenesis in nondiabetic 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 Lampeter *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, 4 years after transplantation of BM from her HLA-identical brother with T1D. At diagnosis of diabetes, the recipient was positive for high-titer 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 Bach (1) to reach this conclusion. The other three criteria are as follows: (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 a 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 and negative selections 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 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 (Fig. 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 Fig. 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 Fig. 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 (nonself) 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

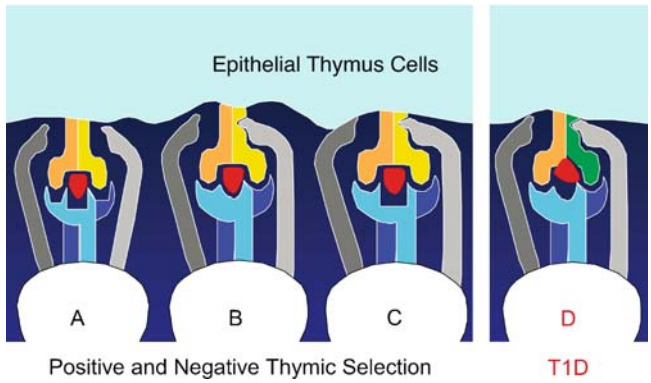


Fig. 1. Theoretical basis of Tian *et al.*'s (62) approach for abrogation of autoimmunity in the nonobese diabetic (NOD) mouse. In the thymus, peptides (in red) from self-antigens are presented to the various (A, B, and C) immature double-positive [CD8 (in gray) and CD4 (lighter gray)] T cells via the major histocompatibility complex (MHC) molecule. When, as for the A cell, the T-cell receptor (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 (nonself) invaders, with which it is able to efficiently interact. In type 1 diabetes (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 *et al.* (62) can be illustrated by imagining that the $I\text{-}Ag^7$ 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 nondiabetogenic MHC molecule, i.e., an Asp-57-positive 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).

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 1980s, 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 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 nonobese 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 has 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 the 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., graft-vs.-host disease (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 (Fig. 2). T1D was prevented by the presence of a "diabetes-resistant" MHC molecule at the surface of hematopoietic stem cells (HSCs) of genetically

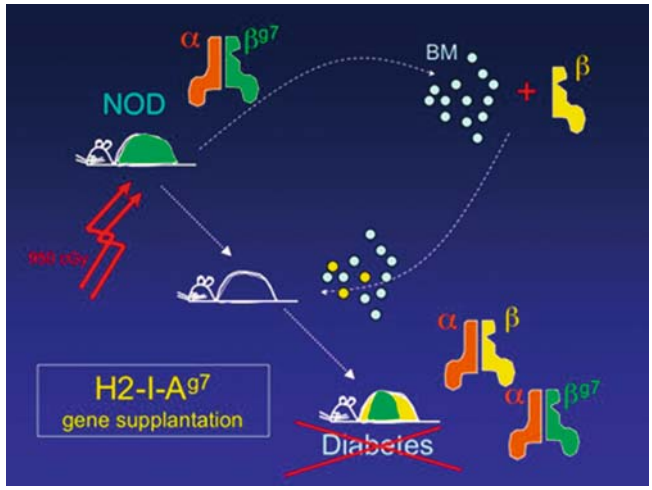


Fig. 2. Schematic representation of Tian *et al.*'s (62) approach for abrogation of autoimmunity in the NOD mouse. NOD bone marrow (BM) stem cells are *ex vivo* transfected with an H-2 I-A beta chain conferring resistance to diabetes (i.e., an Asp-57-positive beta chain, in yellow). The transplanted cells are reinfused in the myeloablated donor that will become a chimera carrying the I-Ag^7 beta chain (in green) able to confer susceptibility and the resistance beta chain. Because 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.

susceptible (i.e., carrying a "diabetes-susceptible" allele) NOD mice. The expression of the newly formed diabetes-resistant molecule in the reinfused hematopoietic cells was sufficient to prevent T1D onset in the NOD mouse even in the presence of the native, diabetogenic non-Asp-57, 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 (APCs) 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 overexpression 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, nonhormone-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 *et al.* (77) who proposed instead that new beta cells can arise only from the preexisting 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 Seaberg *et al.* (73) and Suzuki *et al.* (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 *et al.* (78) at the National Institutes of Health that document the possible transition from epithelial-to-mesenchymal (EMT) cells and vice versa. 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, although 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-regulates insulin and other islet-specific protein expression, but increases 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 2 weeks culture of aggregates under serum-free condition, islet-specific marker expressions, 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.

If a small number of beta cells can indeed undergo EMT, and dedifferentiate into precursor cells, in Dor *et al.*'s (77) pulse-and-chase labeling system, these cells will still be positively labeled. Whereas Dor *et al.*'s explanation inferred a direct replication of beta cells, Gershengorn *et al.*'s (78) data 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 decreases until they become too few to maintain glucohomeostasis 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 to reverse the systematic invasion and inflammation of the islets by autoreactive T cells that results in *insulitis* (58–61).

The allogeneic chimerism induced in prediabetic NOD recipients is multilineage and increases with time: 4 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 composed of both the degree of insulitis, defined by a very detailed scoring system (Fig. 3), and a relative number *A*: the measure of pathology-

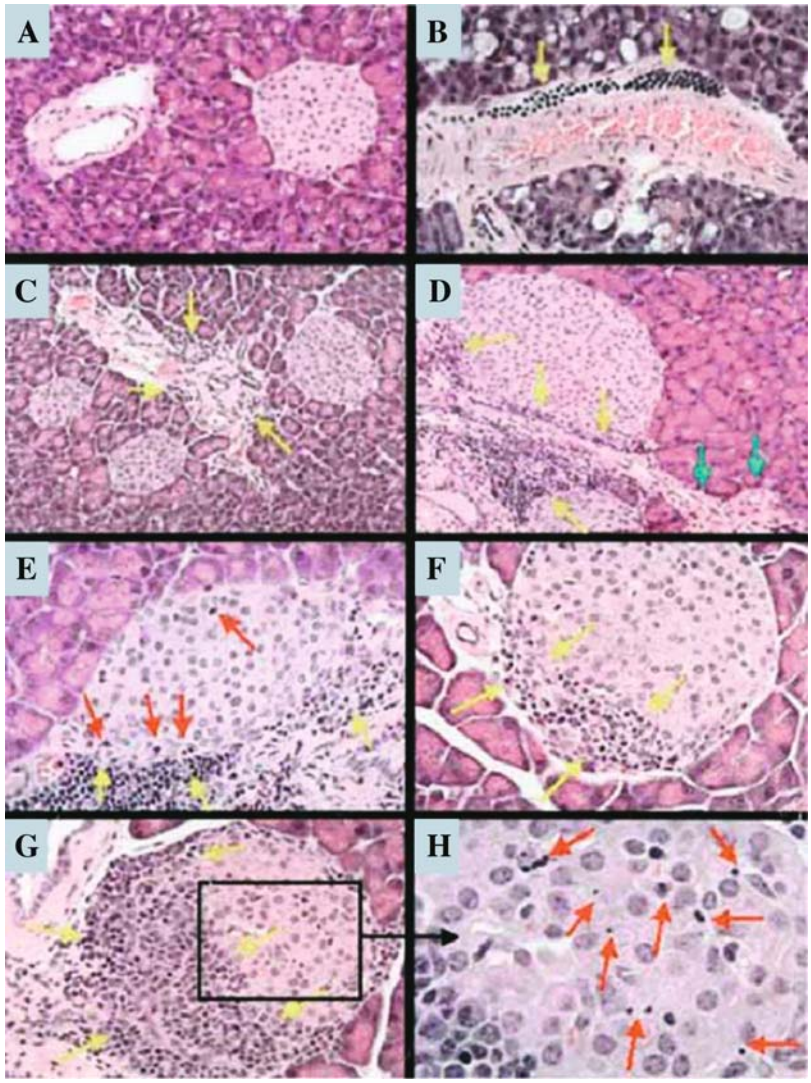


Fig. 3. Scoring of the different stages of destruction of islets of Langerhans during diabetogenesis. Specimens of pancreata from NOD mice of different age were stained with H&E. Magnification for A to G, ×400; for H, ×1000. (A) Score 0: normal pancreatic tissue. Neither morphological abnormalities nor 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).

free area of islet vs. whole pancreatic tissue. The need for this new parameter *A* arose from the observation that in diabetic NOD mice rendered hematopoietic chimeras, a new morphological state of the endocrine pancreas can be recognized. The insulitis-free 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 (Fig. 4). Fourteen

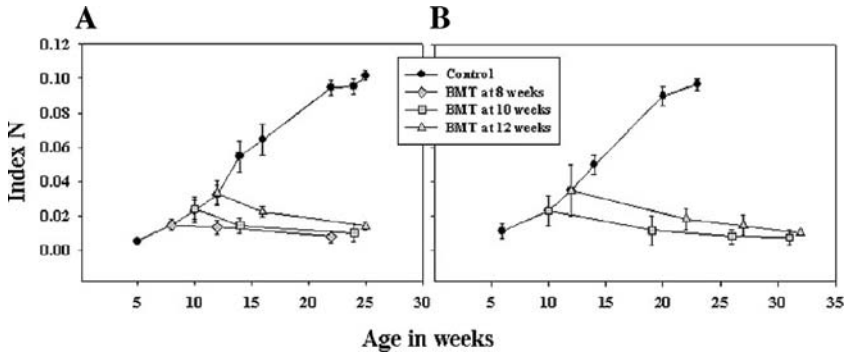


Fig. 4. Chimerism abrogates and reverses destruction of islets of Langerhans in NOD mice prior to the clinical onset of diabetes. NOD mice (8–12 weeks of age) were rendered hematopoietic chimeras by the administration of T-cell-depleted allogeneic BM into recipients conditioned by lethal (A) and nonlethal (B) doses of TBI. Pancreata of these chimeras 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 because untreated animals did not survive long after reaching an Index *N* over 0.1 (61).

weeks after BMT, arrest of the destructive processes and total normalization of Index *N* were observed in all chimeras subjected to nonlethal 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., bromodeoxyuridine (BrdU)-positive] cells can be performed. In the endogenous endocrine pancreas, and in islet allografts of diabetic experimental mice, some

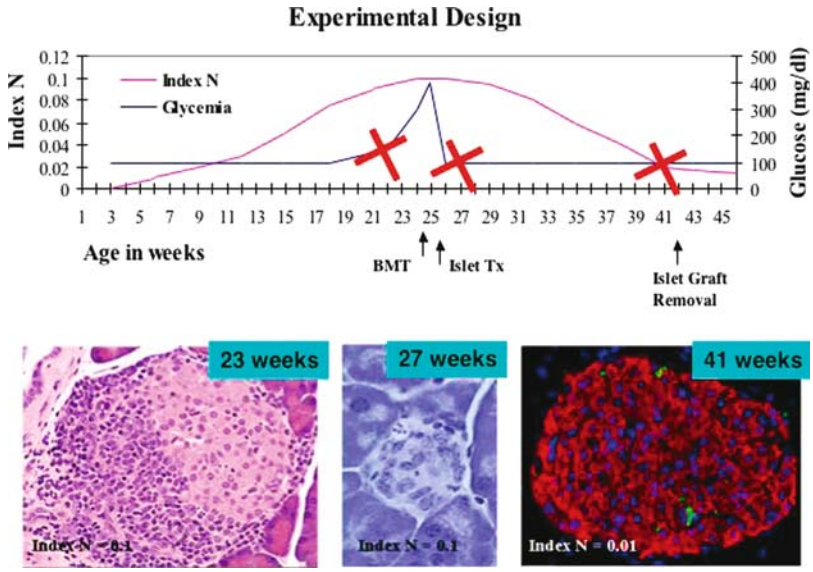
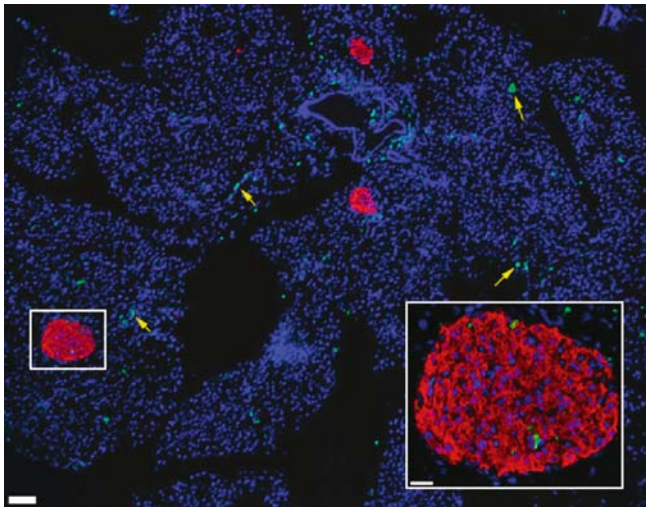
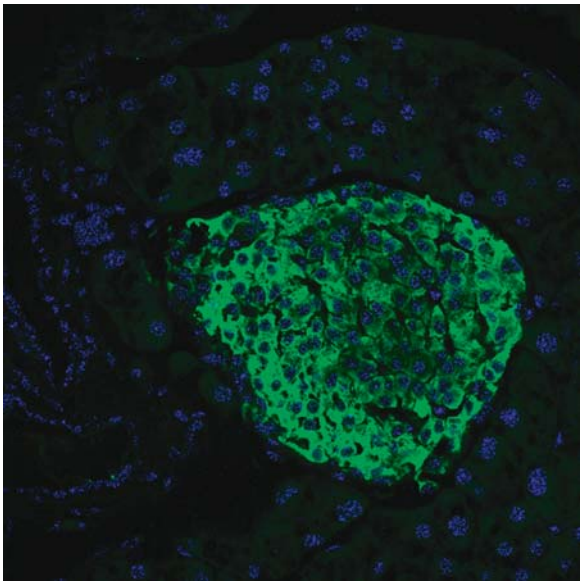
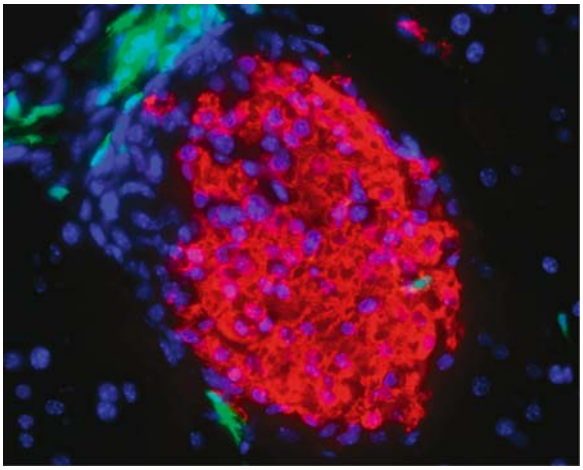


Fig. 5. Schematic representation of the protocol used to test regeneration (or rescue) of the beta cell in diabetic NOD mice. In NOD mice, the infiltration of autoreactive T cells into the islets of Langerhans (resulting in *insulitis*) begins at around 4 weeks of age. At 20–23 weeks, ~85% of female mice are diabetic; that is, their glycemia is >300 mg/dl. When successfully transplanted with bone marrow (BMT) from a nondiabetes-prone donor and hematopoietic chimerism is established, the NOD mouse no longer shows signs of autoimmune activity. However, whereas 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 nondiabetic (61). For "Index *N*" morphometric scoring system, see (60).







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 the onset of the overt disease (61). Spontaneously diabetic NOD mice were rendered hematopoietic chimeras by transplanting them with BM from B6-green fluorescent protein (GFP) mice (81). The rationale for the use of GFP-positive BM cells was to track the fate of donor-derived 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 (Fig. 5). These animals became euglycemic within 24 h 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 (Fig. 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 because insulin-positive cells were not GFP-positive too (Fig. 6b) (45,61). It was actually calculated that, in the cured recipient, insulin-producing cells (that were genetically marked to indicate that 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 cotransplanted 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, Baeyens, and Suarez-Pinzon 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

Fig. 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 nondiabetic animal. Insulin is in red (61). (b) Comparison between an islet of Langerhans of a nondiabetic 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 nondiabetes-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).

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 seems 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 STZ; that is, 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 NONHUMAN 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 interleukin-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 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 1990s 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 toward 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 because, 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 noncalcineurin 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-positive and Glut-2-positive cells disappear in the pancreata of monkeys treated with STZ, but insulin-positive and Glut-2-positive cells reappear after 3–4 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 <0.5 ng/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

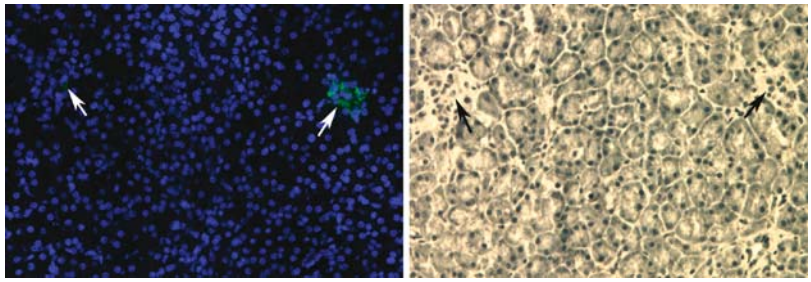


Fig. 7. Newly formed insulin-producing cells in the diabetic monkey. After 3–4 months from STZ injection and diabetes induction, insulin-producing cells are appearing in the monkey endogenous pancreas, eventually forming islet-like conglomerates of insulin-positive cells indicated by the arrows. Immunofluorescence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

least at a detectable pace, because STZ-treated nontransplanted 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 3 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 (Fig. 7; Bottino *et al.*, unpublished observation).

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 Rother and Harlan (15).

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 nondiabetogenic 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 *et al.*'s (62) approach 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 nonfractionated 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 *et al.*'s approach, overtly diabetic (rather than prediabetic) individuals would be treated by the reinfusion 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 the 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 nonhuman primates. Even if nonhuman 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 *et al.*'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 cotransfected into the precursor cells.

For preventing diabetes progression in prediabetic NOD animals by transfected BM precursor cell reconstruction, a combination of AutoMACs with Flow Sorter approaches was used to isolate Sca1-positive, c-Kit-positive, and Lin-negative 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 sublethal radioactive conditioning and showed an evident chimerism in the blood 2 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 because 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 in 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. It is hoped that 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 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, immunoreductive 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 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 Iwakoshi *et al.* (112) in Worcester, consists of 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 GVHD (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 preconditioning 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 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 pheno-

type 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 surfaces 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 because of the presence of regulatory T cells that attenuated these pathologic processes. More recently, Feili-Hariri *et al.* (121,122) have shown prolongation of a diabetes-free state in NOD recipients of BM-derived syngeneic DC. NOD DC exhibits 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, renders 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 that, 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 because intravenous glucose tolerance tests 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 of 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 nonendocrine pancreatic and nonpancreatic tissues seen after i.p. or i.v. delivery, we explored a topical route by retrograde delivery into the pancreatic duct, similar to the endoscopic retrograde cholangiopancreatography technique commonly used in patients with pancreatitis. Because 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 nonspecific 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.

CONCLUSIONS

For decades, efforts have been made to find successful treatments for T1D, such as insulin replacement, pancreas transplantation, and islet transplantation (whether they be

allotransplantation or 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. 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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