Recent advances in molecular and cell biology may allow for the development of novel strategies for the treatment and cure of type 1 diabetes. In particular, it is now possible to envisage restoration of insulin secretion by gene or cell-replacement therapy. The β-cell is, however, remarkably sophisticated, and many of the features of this highly differentiated secretory cell will have to be faithfully mimicked in surrogate cells. In particular, insulin is normally secreted in a well-regulated fashion in rapid response to the metabolic needs of the individual and most specifically (but not exclusively) to changes in circulating levels of glucose. Such regulated secretion will be indispensable in order to avoid both hyper- and hypoglycemic episodes and depends on the ability of cells to store insulin in secretory granules before exocytosis in response to physiological stimuli. Furthermore, any newly created insulin-secreting cell will have to be able to adapt to alterations in insulin requirements that accompany changes with exercise, body weight, and aging. Fine tuning of insulin secretion over the longer term will also be important to avoid “clinical shifting” that could be caused by over- insulinization, including increased adiposity and cardiovascular disease. Finally, it will be necessary to ensure that newly created or implanted (surrogate) β-cells are protected in some way from recognition by the immune system and in particular from autoimmune destruction. Diabetes 50:2181–2191, 2001

Recent success in reversing type 1 diabetes by islet transplantation, as reported by the Edmonton group (1), has led to renewed optimism in the field. It is, however, quite apparent that human islets will never be available in sufficient numbers to satisfy the needs of all the patients in the world with diabetes. Furthermore, islet transplantation still requires long-term (and most likely life-long) immunosuppression. A seductive means of circumventing both of these obstacles would be by gene therapy or by cell-replacement therapy, two possible approaches for replacing endogenous insulin production that we have defined below. Our intention in this article is to place these two methods in as objective a setting as possible and to set minimal standards that we feel need to be met in order for them to be useful in a clinical setting. It is not our purpose to write a comprehensive review of the field, and we do not wish to take sides on any of the issues discussed. Our motivation is straightforward but strongly felt. We are particularly dismayed by the increasing number of papers, more often than not published in highly reputable (and on occasion in widely distributed) journals, claiming to be on track to a cure for diabetes by means of gene or cell therapy. These papers are often misleading, and those misled, given the attention such papers receive in the lay press and on television, are first and foremost patients with diabetes and their families. We encourage everyone to review all papers in this area of research with a critical eye; it is our hope that the present article will be helpful in this regard. That being said, we certainly do not wish to give the impression that these approaches are doomed to failure; rather, we hope that the present guidelines will help speed the effective and safe application of these approaches to the patient.

DEFINITION OF TERMS

Gene therapy. (Insulin) gene therapy will be considered as including any approach that involves the introduction of a foreign gene into any cell type in the body, allowing it to produce insulin. The gene(s) introduced could be the insulin gene itself, perhaps under control of a tissue-specific promoter, allowing for expression in a select non-β-cell type, or a gene encoding a factor that in turn activates the insulin gene, thereby allowing for ectopic insulin production. The induction of stem-cell differentiation into β-cells (or cells with an insulin-producing phenotype) by means of molecular intervention in the patient would be included in this definition of gene therapy. Cell-replacement therapy. Implantation of surrogate β-cells, or the more generic term “cell-replacement therapy,” encompasses all methods that involve the creation or
expansion of insulin-producing cells in vitro followed by their implantation (or reimplantation if from the same individual) in the patient. The cells could be of β-cell origin, and perhaps (conditionally) immortalized to allow for unlimited expansion in culture or non-β-cells manipulated to produce insulin. Alternatively, they could originate from stem cells, whether adult or embryonal, and have been induced to differentiate into β-cells (or selected to this end) in vitro.

HISTORICAL PERSPECTIVE
The mere act of expressing the insulin gene in a non-β-cell is not novel. Indeed, to our knowledge, the first example was that of the expression of insulin in AtT20 (mouse pituitary corticotroph) cells in 1983 (2). Interestingly, this early experiment provided proof of principle that it was possible to synthesize proinsulin in a non-β-cell and have it correctly sorted to secretory granules and converted to insulin, so long as the cell in question was a professional, regulated, secretory cell. It appeared as though any regulated secretory cell type could take care of an ectopically expressed hormone precursor and release the fully processed hormone in a regulated fashion. These data, among others, provided the basis for a landmark review written by Regis Kelly (3) in 1985 that described the salient features of the constitutive and regulated secretory pathways. The implantation of (genetically engineered) surrogate β-cells into (usually streptozotocin-induced) diabetic animals is not particularly innovative either. The earliest reports date from 1987, when Richard Selden et al. (4) described the implantation of fibroblasts expressing proinsulin (driven by the metallothionein promoter) in diabetic mice. Not surprisingly, and for reasons discussed more in detail below, the mice died of hypoglycemia consequent to the excessive and constitutive output of (pro)insulin from the implanted fibroblasts. Interestingly, 1983 also marked the first attempt at insulin gene therapy, with the reported (but admittedly poorly documented) expression of insulin in vivo in animals after the injection of liposome-entrapped insulin cDNA (5).

Since the publication of these early “insulin gene therapy” experiments, many others have been reported in the literature. More often than not, insulin was released in a constitutive fashion, since regulated secretory cells were not used [see for example Chen et al. (6) and Lee et al. (7), to cite but two of the more recent studies], and several learned reviews have been written on the general theme of gene therapy for diabetes [for example (8–11)]. The recent study showing glucose-regulated insulin secretion from a genetically engineered intestinal K-cell line is a refreshing exception in this regard, given the use of bona fide regulated secretory cells (12). Based on the present status of work in this area, it is not particularly comforting or rewarding to be forced to conclude that we have not made much progress in these intervening 18 years; the wheel has been reinvented many times. There is one major contextual difference: finding a “cure for diabetes,” always a worthy academic goal, has now become an industrial goal with high stakes. This leads to increased public exposure in the press of even the most preliminary or primitive studies, but lay readers (and on occasion our own colleagues) are unable to distinguish hype from real hope.

MINIMAL STANDARDS AND EXPECTATIONS
What do we expect of treatment (or ideally, and in common parlance, of a “cure”) for type 1 diabetes by gene therapy or cell-replacement therapy? First, the intervention, regardless of its nature, must result in less morbidity and mortality than the disease itself. To consider one current example, according to these simple criteria, exposing a young child to a lifetime of immunosuppression in order to “cure” diabetes by transplantation is not acceptable. While these issues of safety and ethics are of paramount importance, this is not the appropriate place to discuss them and we are not the appropriate experts. Sufficient to say that we should all be aware of the possible dangers of gene and cell therapy and adopt a suitably humble and conservative approach in the face of so little clinical experience. By contrast, we should be in a position to define quite precisely what we expect of such therapy in terms of the metabolic outcome. Intensive insulin therapy by multiple insulin injections or by an externally controlled (open-loop) insulin pump achieves results that are usually considered quite acceptable by patients and physicians alike. Recent progress in insulin preparations and devices has made insulin therapy more effective and “patient friendly.” Yet, such therapy is not perfect. Reducing hyperglycemia to normoglycemia is inevitably associated with an increased risk of hypoglycemia, and diurnal glycomic excursions are certainly more pronounced than in nondiabetic individuals with second-by-second regulation of insulin secretion. Patients are required to monitor glucose levels frequently, and the injections themselves (or the use of the pump) pose problems of their own. Despite this, most would accept that the continuous introduction of new insulin analogues, as well as improved and increasingly “patient friendly” monitoring and delivery devices, makes existing “standard” therapy quite good. Indeed, such therapy presents a considerable competitive challenge to any innovative therapeutic intervention.

In the following sections, we attempt to describe how clever the β-cell is at controlling glycemia and to define which aspects of β-cell function will be indispensable in order for a non-β-cell to achieve normal metabolic control (in other words, how clever and thoughtful will scientists have to be?). Clearly, in dealing with an autoimmune disease such as type 1 diabetes, the closer we come to imitating a β-cell in fine functional detail, the more likely it will be that the newly created cell will be subject to autoimmune destruction, just as the host’s endogenous β-cells were at the onset of the disease. However, both gene therapy and cell-replacement therapy do allow for the immune characteristics of cells to be modified, to render them less susceptible to autoimmune destruction (or to rejection after implantation), and these issues will also be addressed.

HOW GOOD IS THE β-CELL AT CONTROLLING GLYCEMIA?
The answer to this question is absolutely clear: very good indeed. If given first choice as to which cell in the body to replace by molecular engineering technologies, no sensible scientist would pick the β-cell! Indeed, creating the perfect surrogate β-cell may be a challenge too great for our current state of knowledge and available tools. The
β-cell combines in a single functional unit several remarkable features: regulated transcription and translation (of proinsulin), a regulated secretory pathway (with all its special features), and stimulus-secretion coupling (with exquisite sensitivity most notably but not exclusively to glucose). Together, these features render the β-cell uniquely well suited to its physiological purpose. In this postgenomic era, we are becoming increasingly aware of the combinatorial power of individual genes, proteins, or biochemical pathways. The force of the β-cell thus also lies in a unique combination of functions, with each individual function not necessarily unique to this cell type. Consider two examples. Hepatocytes share with β-cells nonlimiting rates of glucose transport into the cell and glucose phosphorylation predominantly by the high $K_m$ enzyme glucokinase. They are thus well equipped to respond to discrete changes in ambient glucose with appropriate changes in glycolytic flux. Yet, this feature alone will not allow the hepatocyte to become a faithful β-cell surrogate by mere expression of the insulin gene; this cell type has no regulated secretory pathway (even if some hepatoma lines might) (13). Similarly, other endocrine cells and cell lines are endowed with the regulated secretory pathway and can store fully processed insulin in secretory granules, but may lack the stimulus-secretion machinery allowing them to secrete insulin in response to glucose.

INTEGRATED STIMULUS-SECRETION COUPLING CIRCUITRY IN THE β-CELL: A TOUGH ACT TO FOLLOW

In vivo, the β-cell is constantly monitoring metabolic status and is able to generate appropriate secondary stimulus-coupling signals in response to the most minor changes in the concentration of many metabolites, most notably glucose. This is coupled with regulatory input from other signaling pathways, including neuropeptides. Such signals not only lead to well-known alterations in insulin secretion but also influence other (equally important) β-cell functions, including proinsulin synthesis and conversion, insulin degradation, as well as β-cell growth and survival.

Glucose and other fuel secretagogues. Without going into too much molecular detail, it is worthwhile highlighting a few metabolic features of the β-cell that are distinct and enable secondary stimulus-coupling signals to be generated. The β-cell is able to monitor circulating glucose concentrations in the physiologically relevant range (2–20 mmol/L) because it expresses the combination of the "high $K_m$" glucose transporter (whether GLUT2 or another) and glucokinase (14). As previously mentioned, hepatocytes, as well as certain gut cells and hypothalamic neurons, also express these elements, thus enabling them to sense extracellular glucose, but this is a relatively rare feature of mammalian cells. Also characteristic of primary islet β-cell metabolism, but not other mammalian cell types, are very low lactate dehydrogenase and plasma membrane monocarboxylate pyruvate/lactate transporter activities (15), making lactate output in primary β-cells almost undetectable. Consequently, there is a marked increase in mitochondrial metabolic shuttle activities (e.g., the glycerol-3-phosphate shuttle) to reoxidize cytosolic NADH back to NAD⁺, a requirement for glycolysis normally provided by lactate dehydrogenase in most eukaryotic cells. In addition, the primary islet β-cell has several-fold–increased pyruvate carboxylase activity to efficiently direct pyruvate (the major product of glycolysis in the absence of lactate production) toward mitochondrial tricarboxylic acid cycle and oxidative phosphorylation metabolism for efficient ATP production (16). Changes in intracellular ATP production contribute as a key metabolic stimulus-coupling factor in the β-cell to control insulin release (17). The idiosyncratic increased pyruvate carboxylase activity in β-cells leads to glucose-regulated anaplerosis that, in turn, can generate additional candidate metabolic stimulus-coupling signals derived from the tricarboxylic acid cycle, such as glutamate and malonyl-CoA.

Thus, the β-cell has a carefully constructed balance of metabolic enzymes geared up for generating metabolic secondary signals to regulate its function, particularly control of insulin exocytosis, in addition to assuring the energy requirements for its normal day-to-day functions. This is unique among mammalian cells. Consequently, creation of surrogate β-cells with normal or at least adequate glucose stimulus-secretion coupling capacity will not be achievable by merely introducing the glucose sensing capabilities of GLUT2 and glucokinase as well as the insulin gene in cells that possess a regulated secretory pathway. Other downstream metabolic enzymes and the balance between their expression and activities must be considered. In this regard, it is interesting to note that expression of GLUT2 and glucokinase in primary intermediate pituitary cells transgenically engineered to produce insulin caused defective glucose metabolism, glucose toxicity, and apoptosis rather than glucose-induced insulin secretion, as might have been naively anticipated (18).

Other fuel secretagogues (such as leucine) must also be metabolized for their action on secretion to be realized. The metabolites variously generated in this way "plug in" to the metabolic circuitry previously described, leading to the generation of common signals.

Neuroendocrine peptides and other regulators of insulin secretion. There are other influential regulators of β-cell function that should be considered unique to this cell type. First, there is the so-called incretin effect, a communication between the gut and endocrine pancreas that bolsters nutrient-regulated insulin production and secretion. Incretin refers to the peptide hormones glucose-dependent insulinotropic polypeptide (GIP) (also known as gastric inhibitory polypeptide) and especially glucagon-like peptide 1 (GLP-1) (19). GLP-1 receptors are predominantly found on β-cells (and some hypothalamic neurons) leading to the specific glucose-regulated GLP-1-mediated regulation of insulin secretion, proinsulin biosynthesis, and β-cell proliferation (20). Thus, GLP-1 represents another distinctive regulator of β-cell function in addition, and complementary, to nutrient-induced metabolic regulation. The presence of the GLP-1 receptor would be an important consideration in developing surrogate β-cells so that a full postprandial response can be achieved.

Neuronal input can also influence β-cell function and especially insulin secretion. This is of particular importance during episodes of metabolic stress (21). Such neuronal input would almost inevitably be lost in β-cell–replacement therapy.
Convergent downstream effectors. All stimulus-secretion coupling pathways must ultimately converge at a common point, assuring increased exocytosis. The most distal coupling factors or second messengers are thus few in number (including notably Ca\(^{2+}\) and cAMP), and most are probably ubiquitous in terms of their role in all regulated secretory cell types. However, some of the means by which their levels are controlled are unique to each cell type. Furthermore, the mechanism by which changes in effector/messenger levels are translated to increased exocytosis may also be cell-specific. Consider the following examples. Elevation of the concentration of intracellular (free) Ca\(^{2+}\) ([Ca\(^{2+}\)\(_i\)]) can of itself stimulate exocytosis in many (probably all) regulated cell types. Yet, in the β-cell a major pathway leading from glucose stimulation to elevated [Ca\(^{2+}\)\(_i\)], and thus increased insulin secretion depends on the presence of the K\(_{ATP}\)-channel (the target of sulfonylureas). In humans, mutations in the gene for the K\(_{ATP}\)-channel lead to uncontrolled insulin secretion and disease states known collectively as PHHI (persistent hyperinsulinemic hypoglycemia of infancy) (22). There is also convincing evidence for another major glucose-signaling pathway that does not depend on the K\(_{ATP}\)-channel but that nonetheless remains dependent on elevated cytosolic Ca\(^{2+}\) (23) as well as other factors (24). Whereas cAMP can most likely enhance exocytosis of granules in most endocrine cells, the way by which it potentiates glucose-stimulated secretion may reflect processes unique to the β-cell. This particular feature of stimulus-secretion coupling lies at the heart of the ability of glucose to modulate the stimulation of insulin secretion by GLP-1. Finally, we have only recently started to unravel the molecular mechanism of exocytosis itself, and the way this event is ultimately regulated remains to be explored. Components of this last step in insulin secretion may themselves prove to be unique to the β-cell.

The complexity of β-cell stimulus-secretion coupling is already evident, even if not all is yet known. Some features of the system are unique to this cell, whereas others may be expressed in unique combinations. The mere expression of just one or two elements or components in a semi-random fashion will most likely not endow surrogate β-cells with adequately regulated insulin secretion and may prove to be detrimental to cell survival. Searching for ways to achieve an acceptable minimum level of regulation to reverse diabetes without any untoward secondary effects (see below) remains a major challenge.

(PRO)INSULIN PRODUCTION: KEEPING PACE WITH SECRETION

Regulation of insulin synthesis. Insulin gene expression involves a wide variety of transcription factors, some of which interact with one another (25). Furthermore, maintaining preproinsulin mRNA levels depends not only on gene promoter activity, but also on heteronuclear mRNA processing and preproinsulin mRNA stability, the latter of which is likely β-cell–specific. The pancreatic β-cell is able to maintain intracellular stores of insulin at a relatively constant level. When insulin is lost from the β-cell by glucose-induced exocytosis, there is a reciprocally rapid upregulation of proinsulin biosynthesis at the level of transcription (26,27). This is the predominant means of regulating proinsulin production in the β-cell under normal physiological circumstances. However, in the long term such translational control is supplemented by regulation at the transcriptional level and by insulin degradation within the β-cell. Specific glucose-induced translational regulation of proinsulin synthesis appears to be unique to the β-cell, with discrete signals interacting with cis elements in the untranslated regions of preproinsulin mRNA (28). The composition of preproinsulin cDNA to be used for gene therapy vectors thus requires careful attention, but may not itself be sufficient to allow for translational control in a surrogate β-cell.

Conversion of proinsulin to insulin. Proinsulin conversion is mediated by two endoproteases, PC1 (also known as PC3) and PC2, as well as an exopeptidase carboxypeptidase-H (29,30). Conversion arises within secretory granules once they have become acidified and in the presence of the prevailing high intragranular [Ca\(^{2+}\)]. In addition, glucose-induced increase of proinsulin biosynthesis is paralleled by a similar specific translational regulation of the biosynthesis of both PC1 and PC2. It should be noted that PC1 and PC2 are not necessarily required for in vivo proinsulin conversion in other mammalian cell types, so long as the dibasic processing sites on the proinsulin molecule are altered to be recognized by the generic proprotein convertase, furin (31). Under such circumstances, proinsulin is processed, even though it may be secreted via the constitutive pathway (see below). However, conversion under these unnatural circumstances is not typically as efficient as that in the natural setting of the granules of the β-cell–regulated secretory pathway. Furthermore, it remains possible that the mutations needed to make proinsulin sensitive to cleavage by furin may render it immunogenic.

KINETICS OF INSULIN PRODUCTION

Insulin secretion can be stimulated within a minute after exposing the β-cell to a secretagogue. This is the hallmark of the regulated secretory pathway involving discharge, by exocytosis, of insulin stored in secretory granules. The off-response is as rapid. Normal glucose homeostasis absolutely depends on these remarkably rapid kinetics of insulin secretion. We have already discussed stimulus-secretion coupling, which allows for the regulation of exocytosis. Proximal to this, however, lie a number of highly specialized events that together allow for proinsulin to be sorted correctly to nascent granules in the trans-Golgi network (TGN) and for granules to become functionally competent (32,33). Yet, so little is known of these events. In all likelihood, it will not be possible to reconstitute the regulated pathway in a nonspecialized cell until more of the prerequisite components have been discovered. It has been shown, however, that in rat β-cells (and, one presumes, in humans as well) >99% of all newly formed proinsulin molecules are directed to nascent secretory granules and are thus handled by the regulated secretory pathway. It has certainly been known for many years that merely expressing proinsulin itself, even if accompanied by the conversion enzymes PC1 and PC2, does not allow for regulated insulin secretion. Just how many more “regulated pathway” genes would need to be expressed remains to be seen.
Regulated or constitutive secretion? What happens if insulin is not secreted via the regulated pathway? All cells are thought to be able to secrete proteins via the so-called constitutive pathway. This pathway allows the rapid (~20 min) transit of vesicles from the TGN to the plasma membrane followed by immediate and uncontrollable exocytosis (3). The only ready means of regulation is at the level of synthesis. Could cells releasing insulin via the constitutive pathway, but with the most sophisticated regulation possible at the level of gene transcription, ever be satisfactory for reversing type 1 diabetes? Many seem to believe so [see for example (6,7,34-36)]. We do not. Let us examine the facts. Even the most optimistic calculations reveal that stimulating transcription in a quantitatively meaningful fashion takes hours rather than minutes. Furthermore, we are not dealing with a cytosolic protein, but a precursor (preproinsulin) that has to travel through the secretory pathway before exocytosis—this adds an additional 20 min (at the very least) to the minimum time needed from stimulating transcription through to secretion of the first newly formed protein molecules via the constitutive pathway. A realistic estimate for the minimum time between exposing a cell to a stimulus of transcription and secretion of the first proinsulin/insulin molecules synthesized consequent to such stimulation is no less than 2 h. This estimate is borne out by experiments in animals (7). Worse, the “off” response for regulation via transcription is inevitably sluggish, unless the half-life of the mRNA is unusually short, and such is not the case for preproinsulin mRNA. It is true that when preproinsulin mRNA is expressed in hepatocytes its half-life does become shorter (~6 h) than in the β-cell (~24 h) (28), but this time-frame is still far longer than the few minutes required to halt insulin secretion after removal of the stimulus.

As mentioned above, in the β-cell, regulation of insulin gene transcription is normally reserved for longer term, adaptive control. Would it not be more appropriate to attempt to control proinsulin synthesis, when it is expressed in constitutive surrogate cells, at the level of translation? We see problems here as well. First, the precise mechanism of translational control of proinsulin synthesis has yet to be elucidated. Second, even if one could achieve the same level of translational control as seen in the β-cell, it would still not provide kinetics comparable with those seen for regulated exocytosis. For glucose-induced proinsulin biosynthesis there is a 20-min lag period with a peak by 60 min. When a stimulus is removed, proinsulin biosynthesis returns to a basal rate within 90 min. Furthermore, these values are for synthesis per se and not secretion, which even via the constitutive pathway would add more precious minutes to the time-frame.

We are left with our original premise: the β-cell is truly smart. It has a very sophisticated system of stimulus-secretion coupling, tailor-made for the purposes of adjusting insulin secretion on a second-by-second basis to the metabolic needs of the individual. This is combined with the regulated secretory pathway that allows for quasi-instantaneous secretion of exactly the desired amount of stored insulin, independent of the rate of synthesis, followed by the replenishment of insulin stores.

PHYSIOLOGICAL AND CLINICAL CONSIDERATIONS RELATED TO INSULIN RELEASE IN HUMANS: MUST THESE BE ADDRESSED?

Strict metabolic control can be attained with some difficulty using insulin regimens that incorporate either multiple daily injections or insulin pump therapy. To achieve this goal, it is imperative that exogenous insulin be provided in a manner that mimics both of the critical components of endogenous insulin release: basal and stimulated secretion. This fact has been well borne out using insulin pumps for the treatment of type 1 diabetes, where it is known that continuous infusion of insulin at basal levels cannot control the incredible variability of glucose levels found in our patients. Although the goal of strict metabolic control continues to be a challenge for most individuals, with the availability of a number of new insulin analogues, it is becoming more feasible. Thus, if genetically engineered insulin-producing cells are to be contenders in the world of diabetes therapy, they need not only to deliver basal insulin, but also to release insulin on demand, thus bringing glucose levels into line with those seen in healthy subjects. To be truly advantageous, they must do so without the attendant risks associated with exogenous insulin replacement, a very tall order indeed.

Basal or stimulated insulin secretion? Basal insulin release can surely be achieved. However, in many cell types without the appropriate cellular machinery, this is likely to occur largely via the constitutive secretory pathway. While this release may be fairly constant at any given point in time, it is still critical that basal insulin secretion be subject to modulation as it occurs in healthy human subjects. As it is simply basal, at first glance this may not seem to be a critical requirement. However, it is essential that this component of insulin release be able to vary, as normal life events, such as exercise, infection, and weight gain, all require appropriate and potentially rapid changes in insulin output. A failure to do so has the potential to result in hypoglycemia or hyperglycemia. While such events are currently not uncommon in the lives of insulin users, repeated recurrences thereof with gene or cell therapy would not be acceptable.

What about stimulated insulin secretion? To be physiologically appropriate, releasing insulin in response to nutrient intake requires the ability to respond not only to glucose, but also to the building blocks of fats and proteins (37). However, if such were to be achieved, it cannot simply be assumed that all of the pieces of the puzzle are now in place. It is clear that the insulin response to oral ingestion is amplified by contributions from the gastrointestinal tract (38) and is modulated by insulin sensitivity (39). Thus, the incretins, including GLP-1 (40) and GIP (38), will need to be able to increase the gain of the insulin-producing cell if sufficient amounts of this life-dependent peptide are to be released during the physiological state of feeding. While we may not yet fully understand all of the stimulus-secretion coupling mechanisms in the β-cell, by creating a cell with a full complement of the known essential sensing and releasing apparatus, there is a reasonable chance of ending up with a system that is capable of responding to as yet unknown modulators. This is a lofty goal, but certainly a necessary one if we are to cover all of the bases.
Exercise, body weight, and insulin sensitivity. It is clear that \( \beta \)-cell function can be modulated both short and long term. Perhaps two of the best examples are exercise and body weight, the latter a bane of our present society. Exercise has both short- and long-term effects on metabolism. A single bout of aerobic exercise results acutely in enhanced glucose utilization by the insulin-sensitive tissues, which must be balanced by a rapid change in insulin secretion if hypoglycemia is to be avoided. With regular exercise, the physiological changes include both that of each individual acute exercise bout and a longer-term training effect. This training effect involves an enhancement of insulin sensitivity and an associated reciprocal change in insulin secretion, so that glucose tolerance frequently remains nearly identical to that present before commencement of regular exercise. On the other hand, increases in body weight or redistribution of body fat compartments to a more central location require increases in insulin output if glucose tolerance is to remain unchanged. This enhancement of insulin release must occur in response to the reduction in insulin sensitivity that is typically associated with increases in adiposity. However, it is still not well understood how the responsiveness of the \( \beta \)-cell changes so precisely with alterations in insulin sensitivity, as occur with exercise and weight changes. The issue of adaptation via modulation of insulin secretion (or the secretory response) versus changes in \( \beta \)-cell mass is discussed below. Regarding modulation of insulin release, it will be critical to allow for output to be increased more than twentyfold in some insulin-resistant subjects (39). In particular, we need to have at least partial answers to some of the following questions. First, is the \( \beta \)-cell gain of the islet mediated solely via a humoral factor, such as glucose or free fatty acids that act directly on the insulin-secreting cell, or does the central nervous system play a role? Second, if the central nervous system is critical, is the signal from the periphery to the brain humoral or neural? Third, if a component of this process does involve the central nervous system, is there an absolute requirement for neural regulation of the islet? Without reliable answers to some or all of these questions, ensuring the existence of the appropriate sensor(s) in the recreated \( \beta \)-cell may not be a simple achievement and may hamper the ability to recreate normal glucose homeostasis.

Aging and glucose tolerance. A dramatic change in our current approach to insulin therapy must include the goal of improving the quality of life. The other goal clearly must be an improvement in glucose control, which should reduce the ravaging complications of the disease and thereby bring with it the hope of prolonged life. However, prolonging life may in turn create further physiological obstacles for the engineered cell, as it now has to encounter the normal physiology of aging that is associated with a rather interesting change in the regulation of glucose metabolism. For reasons that are still not well understood, healthy aging is associated with a progressive reduction in glucose tolerance. Thus, many older individuals have impaired glucose tolerance or even frank diabetes based on oral glucose tolerance testing. This increase in glucose levels appears to be a compensatory response to a yet unidentified physiological need of the aging process (41) and is achieved by a reduction in insulin release, despite the fact that insulin resistance also accompanies the aging process (42). Thus, we may have to develop approaches that will allow the modified cell to undergo the normal enhancement of insulin output in response to insulin resistance that is required in younger individuals but later in life may allow the same cell to adjust its insulin output in the opposite direction, despite the presence of insulin resistance! Unfortunately, the absolute necessity for such an adaptation is unclear because it is not well understood whether the mild deterioration in glucose tolerance observed in healthy aging is essential in order to accomplish goals such as ensuring sufficient glucose delivery to tissues such as the brain, which utilize glucose independent of insulin. Thus, it is possible that we may well be presenting the engineered system (and ourselves) with another hurdle as a "reward" for prolonging life. If so, it will be important to discover whether overcoming this particular hurdle will be achievable by modulation of insulin secretion alone or whether this will have to be combined with appropriate changes in \( \beta \)-cell mass (see below).

Physiological insulin release and \( \beta \)-cell mass as a physiological adaptive response. It is clear that the mass of \( \beta \)-cells within islets constitutes a complex microorgan that is responsible for fine-tuning the availability and disposal of substrates in the body, glucose being the most well studied and easiest to measure. This complexity is probably for good reason, as the \( \beta \)-cell has a critical responsibility in ensuring the maintenance of metabolic conditions geared toward survival. Although we certainly do not have a full understanding of the mechanisms responsible for this, in designing cell-based therapeutic approaches, it is absolutely essential that we adhere to the strict requirement of a physiologically responsive system. If the cell cannot accomplish such, we will be left with faint hope that redundancies in the system may allow appropriate compensation to occur in a cell that is otherwise not ravaged by the immunological and metabolic abnormalities typically observed in type 1 diabetes.

Assuming that the individual engineered cell can be designed to be physiologically responsive and can modulate its response acutely, a major requirement will have been met. However, when longer-term adaptation is necessary, as is likely in insulin-resistant states such as obesity, an adaptive response of a different nature is likely. Under these circumstances, \( \beta \)-cell mass is increased by both an increase in the size of the individual \( \beta \)-cell and by an increase in the number of \( \beta \)-cells (43,44). Such increased mass helps to lessen the individual \( \beta \)-cell secretory burden. Engineering a cell so that it is capable of varying its insulin release will be a hurdle, but developing one that has the ability to proliferate and to only do so under appropriate circumstances will certainly be quite a challenge. Ensuring that any such proliferation is contained and never leads to hyperinsulinemia and hypoglycemia will be even an greater challenge! Clearly, an alternative would be to remove or destroy some of the implanted surrogate \( \beta \)-cells or to top up the reservoir, according to changing needs. Such fine-tuning in the context of gene-replacement rather than cell-replacement therapy will not be conceivable until much safer means are developed for administering genes to patients. Regardless,
because in real life the fluctuations in secretory demand are likely to vary both on a day-to-day basis and long term, the hurdles presented to the engineered β-cell and to the scientist are enormous.

ARE ANIMAL MODELS APPROPRIATE FOR TESTING THE CAPABILITY OF ENGINEERED β-CELLS TO REVERSE DIABETES IN HUMANS?

Clearly, assessments of the ability of genetically engineered fully responsive "β-cells" to restore glycemic control must be performed in animals before the transfer of this technology to humans. However, when doing these in vivo assessments, it is critical that we not forget the age-old adage that a rodent is not a human in more ways than just appearance.

For good reason, the in vivo work done in this area has until now used rodent models. While this may be useful for certain aspects of metabolism, it has the potential to be misleading for others, including glucose disposal. It is now well accepted that glucose uptake is reliant on at least two mechanisms: insulin-dependent and insulin-independent. Both of these need to be considered when judging the success of any modified cell-based insulin delivery system.

Insulin-dependent glucose disposal is a composite measure of the interaction of insulin sensitivity and insulin secretion, whereas insulin-independent glucose uptake, which has certainly had less scientific focus, is equally, if not more important under different conditions (45,46). In the basal state, a substantial proportion of glucose uptake occurs in the brain by insulin-independent mechanisms, with basal insulin having an important effect to regulate hepatic glucose output (47). The interdependency of these factors and how they may possibly compensate for one another has not been extensively studied. Thus, simply concluding that an experimental approach involving insulin replacement therapy in one model is effective because fasting glycemia is nearly normalized does not necessarily mean that this is going to be true for other models. In addition, after glucose administration, a proportion of glucose uptake into tissues is again insulin-independent (45,46). When using animal models to test new systems, one must keep in mind that the efficiency of glucose uptake by insulin-independent mechanisms in animals is commonly greater than in humans (46); therefore, simply translating animal findings as the likely observation in humans may be risky. Of additional importance is the fact that the early phases of insulin release appear to be vital to restraining the glucose excursion after nutrient ingestion (48,49). Thus, development of a genetically engineered system that lacks these critical features in terms of rapid responsiveness to acute stimulation may well result in dampened enthusiasm when advanced from animals to humans with diabetes. Of course, while advising the application of a degree of caution, we are not suggesting that the course of discovery be retarded. Rather, we seek that the design and application of the approaches undergo vigorous testing to ensure that they are likely to mimic true human physiology before their actual application in humans.

"CLINICAL SHIFTING"—SWITCHING ONE CLINICAL OUTCOME FOR ANOTHER

There is no doubt that the advent of technology permitting life without exogenous insulin replacement and the hypoglycemic and hyperglycemic swings associated with diabetes management will be a giant step forward. It will of course also be of interest to determine whether giving up one clinical problem brings with it another. Some of these can be expected, and others may not have yet been conceived. A few examples, which by the very nature of the state of the technology are not necessarily certain, are illustrative and therefore pertinent.

**Tight metabolic control and adiposity.** The DCCT (Diabetes Control and Complications Trial) has certainly taught us a great deal. In fact, we are still learning a lot from the experience. One unpredicted outcome has been the observation that some members of the study cohort that were randomized to and achieved strict metabolic control gained large amounts of weight, whereas others did not. It is becoming evident that there has been "clinical shifting" in the phenotype of these individuals to embrace features of type 2 diabetes, including central adiposity and an adverse lipid profile (50). Could this have been prevented? Probably not. However, it does appear that the individuals in whom this occurred had a family history of type 2 diabetes, so in the future it may be more predictable. These findings present another myriad of issues that will require clinical therapeutic attention and at the same time impose on the engineered β-cell the essential requirement of further modulating insulin release to metabolic need.

**Insulin resistance, proinsulin, and cardiovascular disease.** Cardiovascular disease has been associated with insulin resistance. Some of this may certainly be associated with obesity and particularly central adiposity. However, while the debate rages on as to whether insulin resistance is a risk factor and whether hyperinsulinemia is deleterious for health, it would seem prudent at this point in time to try to avoid excessive insulin exposure, if at all possible. Thus, we will need to try to avoid "clinical shifting" from a state of increased risk of cardiovascular disease as a result of the metabolic derangement associated with hyperglycemia, to a state of increased cardiovascular disease associated with insulin resistance. To ensure that this does not occur, an engineered β-cell that is capable of releasing insulin in a pulsatile fashion may be required, as continuous nonpulsatile administration of insulin is more likely to result in iatrogenic insulin resistance (51,52). Similarly, administration of excessive insulin not only carries the risk of hypoglycemia, but at the same time results in the downregulation of the insulin receptor and thus the development of impaired insulin responsiveness.

The clinical experience with proinsulin as a therapeutic modality raises another specter related to cardiovascular disease. The insulin precursor molecule has been demonstrated to be effective in lowering glucose in type 2 diabetes (53). Unfortunately, however, a clinical trial with proinsulin had to be discontinued because of a possible increase in cardiovascular mortality. That said, the true clinical significance of the data from this particular trial and the mechanism by which a potential increase in
mortality with proinsulin may occur remain unclear. However, in vitro studies have suggested that proinsulin is capable of enhancing the production of substrates that are associated with an increased atherogenic risk (54). Because proinsulin is a normal secretory product of the human β-cell (55), it is quite possible that gene or cell-replacement therapy may be associated with the release of proinsulin into plasma (with increased amounts if proinsulin conversion is not quite as efficient as in the native β-cell). What we need to do is ensure that the amount is not excessive, for fear that if proinsulin is indeed atherogenic, we may simply create "clinical shifting," by trading the etiology of cardiovascular disease in type 1 diabetes from one cause to another.

**Immunosuppressive therapy, malignancy, and other adverse effects.** At the present time, nearly all instances of islet or whole-pancreas transplantation necessitate the use of immunosuppressive therapy to prevent rejection of the transplant. These approaches are not without problems.

Although the "Edmonton" protocol (1) avoids the use of glucocorticoids, they have been the mainstay of regimens used for suppressing the rejection process. These agents produce insulin resistance de novo (56) and at the same time decrease islet endocrine function (57,58). In many instances, the β-cell is incapable of increasing insulin release sufficiently in response to such insulin resistance, and hyperglycemia ensues. Clearly, the challenge for the future, as genetically engineered approaches are developed and tested, is to ensure that β-cells are capable of responding to the pharmacological effects of glucocorticoids, because these agents (or other drugs with similar side-effects) may continue to be required to prevent rejection of other transplanted organs or perhaps to treat various other diseases. The alternative task will be to develop approaches that avoid agents such as glucocorticoids altogether. The number of new immunosuppressive agents currently in clinical trials is impressive; however, possible effects on β-cell function will need to be tested.

At the present time, transplantation is largely limited to individuals who have had long-standing diabetes and who frequently have underlying renal dysfunction. Exposure to immunomodulators, such as cyclosporine, FK 506, and tacrolimus, has been associated with a deterioration in renal function manifested as a reduction in creatinine clearance and an increase in plasma creatinine (59,60). This change in renal function is commonly associated with altered glucose metabolism, including a change in insulin kinetics. Thus, future approaches must include immunosuppressive agents that do not adversely affect renal function and/or genetically engineered systems that can respond appropriately to alterations in the kinetics of insulin, if such agents are necessary. The fact that insulin is an integral part of the immune response is often overlooked and certainly understudied. The appearance of insulin receptors on activated T-cells and the marked insulin requirements during infection would be additional demands on a genetically engineered system.

Lastly, and perhaps most importantly, the use of immunosuppressive therapy can be associated with "clinical shifting" in one of the worst ways. While these agents have revolutionized transplantation and helped the cause of many individuals with diabetes, they have also been associated with the development of malignancy (59,60). It can clearly be argued that in many instances this risk is worth taking. However, if gene or cell-replacement therapy is going to be offered as a more routine procedure, then the risk of iatrogenic malignancy has to be markedly reduced, if not completely eliminated. One way to reduce the risk will be to transduce cells taken from the patient and, after the delivery of genes, give these cells back to the same individual. Although this individualized approach to treatment may reduce or eliminate the need for immunosuppression, it does not fully eliminate the risk for iatrogenic malignancy. Thus, as detailed below, further development of clinical systems the immune response in essence no longer comes into play while avoiding any "clinical shifting."

**THE IMMUNE SYSTEM AND GENE OR CELL-REPLACEMENT THERAPY**

The poor outcome of the numerous attempts to transplant human islets during the past quarter of a century is interpreted to be caused by a host of factors; one is allerejection. Despite long-term immunosuppression, insulin production in type 1 diabetic patients with transplanted islets has historically been transient at best. State-of-the-art immunosuppression that prevents allorejection of kidney, heart, or liver seems to fail with human islets. Transduction of cells from the patient per se would at least alleviate this problem. Indeed, the best results of islet transplantation are seen with autotransplantation of islets obtained from the patient's own pancreas after resection for chronic pancreatitis (61). The long-term function of autotransplanted islets suggests that perhaps current immunosuppressive regimens harm transplanted islets. Interpretation of these results is of course complicated by the fact that the autotransplanted islets in these particular clinical circumstances are, in addition to not being exposed to allorejection, not subject to the autoimmune attack that is the peril in type 1 diabetes. The complexity of dealing with three different immune attacks, i.e., acute rejection, chronic rejection, and recurrence of the disease, has drastically hampered clinical islet transplantation. In addition, there is a wealth of in vitro data suggesting that β-cell function is affected by cyclosporin, prednisone, or azathioprine. As promising as the early results may be, the possible effects on β-cell function and survival of the glucocorticoid-free immunosuppression regimen used in the "Edmonton" protocol in the longer term remain to be determined (1,62). The last immune peril of islet transplantation is the most compelling. Despite immunosuppression and matching for HLA, there are several studies suggesting that the loss of function of transplanted islets was caused by recurrence of disease, as was amply demonstrated in pancreas transplantation between monozygotic twins (63). Failed islet transplants have been reported to be associated with reappearance of GAD65 autoantibodies, a major serological marker for type 1 diabetes (64).

The immune system has a fabulous memory, and this ability will haunt the gene therapist who is trying to grow β-cells, perhaps from stem cells in the pancreas or reintroduced after extra corporal transduction and manipulation. There are as many ideas and possibilities as there are...
scientists working on a way to fend off the immune system from re-attacking the β-cells. Certainly, these cells are in desperate need of a shield to ward off immune attack. The idea of developing encapsulated islets is not new. Most would agree that the current state of this art (for such it is) still falls short of the stringent requirements of clinical application. It is to be hoped that further research will result in capsules or synthetic immune barriers that show long-term function, appropriate diffusion properties, and adequate biocompatibility. Although insulin may be able to leave the barrier to help control blood glucose, it will also be necessary to take into account the entry of factors such as cytokines that impair β-cell function. Genetically engineered immune barriers are also being pursued with the idea to transduce islets or islet cells with factors that fend off immune attack. The consequences of local production of cytokines or chemokines at the site of islet implantation and for the maintenance of long-term β-cell function are but a few of many unknown factors. The basic mechanisms by which the β-cells are killed by the immune system have not been worked out in detail. Thus, we are reduced to trial and error in the search for genes that code for factors that would make the immune system blind to transplanted islets or transduced insulin-producing cells. A confounding issue is that these experiments may be truly informative only in the human system and that the translation from the NOD mouse or the BB rat to humans will be poor at best and most likely irrelevant to human type 1 diabetes. Indeed, the many ways that the NOD mouse can be cured have been shown to be poorly applicable to human type 1 diabetes (65).

At first sight, it could be thought that gene therapy is not applicable to type 1 diabetes because there is no single faulty gene that needs to be corrected. However, molecular manipulation of transplanted islets or cells to shield them from immune attack to avoid allorecognition, recurrence of disease, or both is certainly an attractive proposition. It will be necessary to proceed with caution because, as indicated above, the β-cell is peculiarly sensitive and delicately regulated; the expression of novel genes may lead to undesirable effects on cell function. The alternative of taking cells such as hepatocytes from the recipient to be reintroduced after in vitro transduction should circumvent the risk for allorecognition but does not exclude the recurrence of disease in the event that the patient's immune system targets insulin as a major autoantigen. The importance of insulin as a target is highlighted by the fact that at the time of clinical diagnosis of type 1 diabetes in children <10 years of age, as many as 60% may have insulin autoantibodies (60). β-cells are most likely killed by cytotoxic CD8 T-cells that recognize their target through class I HLA molecules presenting short peptides on the cell surface. These short peptides are loaded within the cell, and a liver cell transfected with the insulin gene is likely to express class I molecules loaded with insulin peptides. This possible scenario needs to be taken into account when contemplating transduction experiments with cells taken from recipient type 1 diabetic patients. Recurrence of the T-cell attack will also need to be considered when turning cells such as hepatocytes into insulin-producing cells with systemic approaches of gene transduction. And, when these factors appear to be at hand, it must be remembered that although preclinical investigations in the NOD mouse and the BB rat may provide proof of principle to please the stock market, they are less likely to provide suitable measures of preclinical safety for future clinical trials.

CONCLUSIONS
To those who see our standards as having been set too high, we answer that the β-cell and the peculiar immunological and metabolic constraints of type 1 diabetes set the standards, not us. We are convinced that in order to treat type 1 diabetes by gene or cell-replacement therapy, without the need for any other adjunct insulin therapy, it will be essential to have well-regulated insulin secretion. Constitutive secretion, regardless of the accompanying degree of regulation of insulin gene transcription, will not achieve the desired result in patients with type 1 diabetes. Clearly, secretion itself must be further regulated by appropriate stimuli, including if possible glucose. Introducing glucose-sensitivity to otherwise insensitive cells may be more complex than previously imagined. It will be imperative to assess cell function carefully before any clinical application. Although the most recent immunosuppressive regimens are less toxic to insulin-secreting cells than more traditional ones, it is to be hoped that greater understanding of the molecular mechanism of both allograft rejection and autoimmune destruction of β-cells will allow for genetic engineering of cells resistant to both. Finally, it is self-evident that new and more plentiful sources of insulin-secreting cells will be needed to treat type 1 diabetes. The most recent (albeit very preliminary) developments in stem cell research are promising in this regard, with islets or islet cells having been derived from both embryonal murine (67,68) and embryonal human (69), as well as adult human pancreatic ductal stem cells (70).

What then are the prospects for successful gene therapy of type 1 diabetes? We are cautiously optimistic. Success, however, will depend on respect for some basic rules of engagement as well as improved understanding of certain key events. Let us hope that the rate of progress and discovery in the needed areas will be brisk.

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