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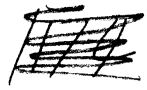

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ISLET ISOLATION ASSESSMENT IN MAN AND LARGE ANIMALS

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The treatment of diabetes mellitus by transplantation of isolated pancreatic islets is an approach that remains the subject of research by a large number of investigators throughout the world. A crucial requirement for the success of this enterprise is the ability to prepare viable isolated islets in adequate quantity. Over the years numerous descriptions of procedures for islet isolation from the pancreas of experimental animals and of man have been advanced; each claiming to be an improvement on previous methods. Indeed, there certainly have been advances, although few techniques live up to the claims that are made in their support. Part of the problem is the generally poor methodology

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used to assess the outcome of islet isolation techniques. For a scientific subject to advance it is necessary to have accurate methods of measurements, for otherwise comparisons of the results obtained by different investigators are impossible.

The problem is less for techniques described for isolation of islets from the pancreas of small animals such as the rodent, where it is possible to actually count the number of islets retrieved, although even here many errors are possible and have undoubtedly occurred. Recently there have been reports of increasingly successful islet isolation from the pancreas of larger mammals and man, where the yields obtained have been too large to count directly. This progress has accentuated the need for the development of precise and reproducible techniques for assessing islet yield in use in different laboratories. The variety of quantitative assessment techniques has made meaningful comparison of results very difficult and the situation is further compounded by similar difficulties facing such issues as the purity and viability of the preparation.

With the above difficulties in mind, a workshop was organized at the 2nd Congress on Pancreas and Islet Transplantation to develop a consensus on criteria for assessment of islets isolated from the pancreas of large mammals, including humans. Both quantitative and qualitative criteria were discussed, and the discussion focused on the main topics of assessment of islet number and volume, the purity of the preparation, morphologic integrity, and test of islet viability *in vitro* and *in vivo*.

Islet number, volume, and purity - The sampling technique represents a critical factor that could affect the results of an islet count as well as insulin content from any islet preparation. Since islets settle rapidly in any container, care must be taken to properly suspend the preparation before sampling to ensure a representative sample. It is preferable also to collect multiple samples to minimize counting errors (e.g. 5 aliquots of 50 μl after suspension of the islet preparation in a 200 ml flask). If the preparation is not needed for transplatation it is also wise to collect a larger sample that may be more representative (e.g. 1 ml from 200 ml islet suspension).

Even though experience facilitates the ability to distinguish an islet from a clump of exocrine cells, ganglia, lymph nodes or so-called 'membrane balls' at the light microscopical level, it has been uniformly recommended that a specific stain for immediate detection of islet tissue should be used in any sample. For this purpose dithizone (DTZ) seems to be the most appropriate. An easy and rapid way to stain islets is to add few drops of a DTZ solution [prepared as follows: add 50 mg DTZ to 5 ml dimethylsulfoxide (DMSO), stock solution, and dilute 1 ml of this solution with 20 ml of Hank's with 2% fetal calf serum (FCS) to a sample contained in a petri dish]. The solution must be freshly prepared and filtered before use. After staining the sample it is possible to count the islets that will appear red as previously described[20]. Since islet volume is approximately proportional to the cube of the islet radius, it is crucial to divide the islets into diameter classes. For this purpose it is possible to use a calibrated grid in the eyepiece of the phase contrast microscope[24]. As a matter of routine, most workers use 50 μ diameter range increments without considering particles smaller than 50 μ, since their contribution to the total volume of the preparation would not be significant. Tab. 1 indicates the mean volume for

each diameter class and the relative conversion factor into islets of 150 μ diameter. These factors make it possible to convert the total islet number from any preparation into equivalent islet number (EIN). In place of EIN, the total islet volume of the final preparation can also be estimated. Nevertheless, EIN can be useful for those who are not familiar with conversion of the volume of a preparation into islet number. As a general rule, the more descriptive the results of an isolation are, the better their interpretation. The potential use of automated methods using computerized imaging analyzers appears to be an attractive alternative to visual methods and may provide a more objective and standard quantitative evaluation. Although this technology appears very promising, it is expensive and judged to be too premature to propose it as a general standard.

Although many approaches for determination of islet purity have been proposed, including insulin/amylase ratio and algebric equations, each has inherent problems that result in assessment variabilities that are not easily controlled. Again, it is possible that the automatic methods employing either specific stains or highly specific monoclonal antibodies will be used ultimately, but for now most investigators use the dithizone stain described earlier as an easy method to roughly estimate the approximate degree of purity of the islets in any preparation.

Morphological assessment - The appropriate morphological assessment of isolated islet preparations should include three different aspects: confirmation of islet identification, assessment of the morphological integrity of the isolated and purified islets as well as the assessment of the purity of the islet preparation.

While several recent papers have focused on the critical assessment of number, volume, purity and function of isolated islets, there have been few studies that have explicitly focused on the detailed morphological investigation of isolated islets[35, 39].

Many studies reporting improved methods for islet isolation lack adequate information demonstrating that the structures identified as islets by stereomicroscopy were, in fact, islets. Although dithizone staining[20] of wet islet preparations is to be seen as a fairly specific method of islet verification (e.g. bovine

islet diameter range (μ)	mean volume (μ^3)	conversion into islets of 150 μ diameter
50-100	294,525	n/ 6.00
100-150	1,145,373	n/ 1.50
150-200	2,977,968	n× 1.7
200-250	6,185,010	n× 3.5
250-300	11,159,198	n× 6.3
300-350	18,293,231	n×10.4
350-400	27,979,808	n×15.8

Tab. 1 - Determination of islet volume for each 50 μ diameter range and relative conversion into equivalent number of islets with a diameter of 150 μ.

islets remain unstained after adding dithizone), the accurate identification of islets by stereomicroscopy should therefore be confirmed by subsequent light microscopic examination demonstrating positive aldehyde-fuchsin staining or immunoperoxidase insulin-labeling of B-cells or, on a more complex level, by electron microscopic evaluation showing characteristic granules within endocrine cells. Another method that permits rapid, accurate, and simple confirmation of islet identification within 3-4h is by double antibody indirect immunofluorescence of cryostat sections, in which a guinea pig unlabeled insulin antibody is reacted, in a second step, by application of fluorescein-labeled goat-antiguinea pig immunoglobulin. The accurate identification of islets employing either immunocytochemical staining or electron microscopic examination should be an essential inclusion in every newly reported method of islet isolation and purification.

After islet identification has been convincingly established, it is important to evaluate the morphological integrity of the islets that were isolated. Many of the available viability tests fail to exclude the possibility that the islet, as a microorgan with a subtle and complex organization, has been seriously injured. Surprisingly little is known about the effects of pancreas procurement, islet isolation, islet purification and islet banking procedures on the morphological integrity of the islet tissue[1, 11, 17, 23, 25, 26, 30, 32, 43, 44]. The relative paucity of studies dealing with the morphology of isolated islets probably reflects a complacent attitude that developed early after experience indicated that isolated rodent islet tissue was well preserved and that islet tissue appeared to be much more resistant to damage during the isolation procedure than acinar cells.

However, more recent studies have emphasized major differences in the structure and integrity of islets isolated from rodents and dogs[1], indicating that the isolation procedures applied in large animals disrupt the vascular, neural and paracrine relationship components of the native islets, potentially affecting important functional interrelationships between islet cell populations. ALEJANDRO et al.[1] demonstrated that while rodent islets remained compact after isolation with their margins predominantly smooth and tightly epithelialized, isolated canine islets were, in contrast, frequently fragmented and the endocrine cells appeared to be loosely adherent to each other. This observation particularly applies to islets retrieved from the porcine pancreas. RICORDI et al.[30] pointed out that the loose organization of islets within the pig pancreas probably accounts for their marked fragility and rapid dissociation into single cells during the isolation procedure. It is interesting to note in this context that one can be surprised by discrepancies between the seemingly intact stereomicroscopical appearance of isolated porcine islets and the abnormal morphological features of the same islets in paraffin or semithin sections. It is highly desirable, therefore, that newly published studies on islet isolation and purification include a detailed analysis of the morphological features. In particular, attention should be paid to the signals of traumatic damage such as irregularly shaped or fragmented islets, discontinuous plasma membranes, mitochondrial swelling with inner membrane disruption, cytoplasmic vacuolation due to massive dilatation of cisternae of the endoplasmic reticulum, reduction of cytoplasmic and nuclear matrix density or pyknotic nuclei.

In addition, it is important to determine whether the individual islet cells are randomly scattered and loosely adherent or whether they are still organized in relation to each other corresponding to the normal islet architecture within the native pancreas.

Another significant issue is whether the connective and vascular tissue within the islets has been preserved and the isolated islets are free or are surrounded by connective and exocrine tissue. In this context, scanning electron microscopy[15, 46] as well as confocal microscopy, a recently described technique for analyzing the three-dimensional structure of isolated islets[7], will be considered as additional approaches to study surface changes of isolated islets.

It is important for co-workers in the field to examine representative photomicrographs of stained paraffin or semithin sections so as to enable them to independently evaluate the extent of the purification (ratio of islet to non-islet tissue). The use of electron microscopy routinely to assess purity is acknowledged to be difficult[28]. The procedures most commonly used for the embedding of islets require sedimentation, and the pellet that is formed generally does not contain randomly distributed cells. In addition, the frequency of islet or non-islet tissue may depend upon what part of the block was assessed.

Viability assays - Viability assays, at best, are indirect measures of cellular anabolic properties such as membrane integrity, nucleotide incorporation, and enzyme content; or catabolic properties such as respiratory quotient. Quantitative tests of pancreatic islet viability, including measurements of insulin biosynthesis, insulin secretion, and respiration have typically been used in studies of basic islet physiology, pathology, and responses to pharmacologic agents. Less elegant (but also less labor-intensive) tests of islet viability involve assessment of membrane permeability. However, such tests can be rapidly performed, an advantage if used to assess islet viability just prior to islet transplantation.

Chromogenic dyes such as neutral red (inclusion of dye by live cells) or trypan blue (exclusion of dye by live cells) are of limited usefulness since live cells or dead cells can be visualized, but not both. The use of fluorometric inclusion and exclusion dyes together have been used to simultaneously quantitate the proportion of cells that are intact or that are damaged. For example, fluorescein diacetate (FDA), a nonpolar ester, freely passes through the cell membrane of live cells. Within the live cell it is ultimately hydrolyzed to the polar free fluorescein, where it is trapped within the intact membrane[12]. Acridine orange, a monovalent, cationic dye, is also membrane permeable. It binds to nucleic acids, and in low concentration causes a green fluorescence in living cells. The exclusion dyes propidium iodide, or the parent analog ethidium bromide, cannot penetrate living cells, but readily enter dead or dying cells. They intercalate with DNA or RNA and form bright red fluorescent complexes.

The combination of inclusion and exclusion dyes acridine orange (AO) and propidium iodide (PI) have been extensively studied as an assay of islets viability[5, 6]. The dyes have minimal background fluorescence, and when used in optimal concentrations[1] (AO – 0.67 μmol/l; PI – 75 μmol/l) stain living cells green and dead cells red. Utilizing this fluorometric method, viable and

non-viable whole islets may be differentiated, as well as viable and non-viable components within an islet. The stability, cytotoxicity and reproductivity of the assay has been demonstrated on isolated islets derived from mice, rats, dogs, and humans.

In vitro *studies* - The isolation of large numbers of pure human islets is not the only consideration for successful transplantation studies. The isolation process itself may alter the functionality of the islets. It is thus crucial that the isolated islets be shown not only to be viable but also able to respond appropriately to glucose stimulation. *In vitro* biochemical techniques have predominantly been used to assess islet viability. Measurement of insulin release from islets after stimulation with different concentrations of glucose has commonly been used in static incubations[2,3,4,11] or in a continuous system by 'perifusion'[16,30,34,37]. Glucose utilization[3], protein (insulin) synthesis[14,34] and oxygen utilization[34] have also been used to assess vital islet functions.

Perifusion of islets with glucose provides a dynamic profile of the characteristics of glucose-mediated insulin release from pre-stored and newly-synthesized insulin and of the ability of the cells to down regulate insulin secretion after the glycemic challenge is interrupted[45]. The results of perifusion of large mammal or human islets depend upon many factors. Factors affecting the dynamic secretion of insulin by isolated islets are the procurement technique for the pancreas, including the nature of the preservation solutions, the duration of hypothermic storage[44] and the properties of the gradient materials used during islet purification (step with Percoll or Ficoll)[29]. The size of the islets selected also influences the quantity of insulin secreted per islet[24]. A preliminary period of tissue culture following isolation or cryopreservation can stabilize basal insulin release[17,26]. All of these factors should be reported in studies that use perifusion as an index of islet viability. Perifusion protocols which yield consistent results are based upon a method reported by LACY et al.[16] in 1976. Duplicate groups of 200-300 islets of comparable size are transferred to millipore chambers and suspended on a filter of 5.0 μm. The chambers are closed, care being taken to exclude all air. They are inserted into an incubator at 37 °C connected to a pump that delivers perfusate at 1.0 ml/min and PE50 outflow tubing that passes to collection vials which rotate on a fraction collector. The perfusate is Krebs-Ringer bicarbonate solution which is maintained at 37 °C and gassed with 95% O_2:5% CO_2. The perfusate is pumped through the chambers for three consecutive hours, during which the glucose concentration is 50, 500 and 50 mg/dl, respectively. The effluent from the chamber is sampled every 10 min after subjecting the islets to perifusion for 40 min to eliminate an artifactual rise in insulin due to mechanical stimulation from the transfer procedure. During the second hour, samples are removed at 1, 2.5, 5, 7.5, 10, 20, 30, 40, 50 and 60 min, and during the final hour every 10 min. The samples are collected at 4 °C and stored at –20 °C until assay for insulin using a double antibody radioimmunoassay technique[9].

Standards for reporting results of perifusion data are critical for the accurate comparison of data. The minimum values that should be reported are the absolute levels of insulin secretion during the pre-challenge basal period, the first 10 min (first phase) and last 50 min (second phase) of high glucose chal-

lenge, and the last 30 min after return to low glucose. The profile of insulin release is best reported as a plot that shows the release during the three consecutive periods. An acceptable way of interpreting data would be to report 'stimulation indices'[44] estimated by determining the ratio between basal (last 15 min before high glucose and last 15 min after return to basal conditions) and stimulated insulin release (first 15 min and last 15 min of stimulation). This method of reporting identifies the secretory capacity but lacks details of basal insulin release, the quality of the biphasic response and return to basal secretion, it should not, therefore, be reported alone. For convenience of comparison of insulin release data from various protocols within a given study, secretion can be assessed independently of basal secretion values by subtracting mean pre-challenge basal levels from the remaining values to show the incremental insulin secretion[29].

Although perifusion data provides a useful guide to islet viability, the quantity and kinetics of insulin release do not necessarily predict islet performance after implantation. For example, rodent islets cryopreserved with slow cooling and thawed with slow warming showed a similar perifusion response to islets cooled slowly and thawed rapidly, but the former did not reverse streptozotocin diabetes[24]. Similarly, frozen-thawed purified canine islets failed to secrete insulin during perifusion, but they consistently induced normoglycemia after autoimplantation into the spleen of pancreatectomized recipients[10]. Therefore, the ultimate test of viability is functional activity after transplantation into a diabetic recipient. This tests not only insulin secretory capacity, but also viability that allows the islet to withstand the rigors of engraftment in an ectopic site of a diabetic recipient until revascularization is complete.

In vivo *studies* - Attempts have been made to ascertain human islet viability *in vivo* by transplantation into a specific animal model, the nude (athymic) rodent. These animals have a deficient immune system due to congenital thymic aplasia and are unable to reject transplanted xenogeneic tissue[24]. The first report of transplantation of human pancreatic tissue into the nude rodent was by POVLSEN et al.[27] in 1974, when several portions of human fetal pancreas were transplanted subcutaneously into a non-diabetic nude mouse. Histological examination of the excised tissue at 64 days post-transplantation revealed a relatively normal lobular appearance with no sign of rejection. A number of groups subsequently reported further success with transplantation of human fetal pancreas into the non-diabetic nude mouse with observation of histological differentiation and the maturation of endocrine tissue[10, 22, 27, 42].

Viable insulin containing tissue with no sign of rejection was also demonstrated histologically, at 2 weeks post-implantation, in four survivors out of 15 non-diabetic nude mice transplanted with freshly isolated human adult islets by GRAY et al.[11] and more recently, in the nude rat, with cryopreserved human islets[43]. Although these reports indicated the potential value of this animal model for xenotransplant studies, the assessment of the functional ability of transplanted tissue is only possible in diabetic recipients.

BUSCHARD et al.[8] were the first to report reversal of streptozotocin-induced diabetes in 3 out of 6 nude mice transplanted subcutaneously with 1-3 neonatal rat whole pancreas grafts. Blood glucose returned to normal 2 weeks after

transplantation and the animals survived and gained weight for 27-93 days. Intraperitoneal transplantation of isolated human islets (2 cases) and allogeneous mouse islets (4 successes out of 8 transplants) into diabetic nude mouse was subsequently reported[23, 34]. As indicated above the criteria for success was a reduction in blood glucose over a period of time. Glucose levels did not, however, return to normal and there was no confirmation of graft function by glucose tolerance testing, reversal to the diabetic state following graft removal or histological confirmation of graft survival. The maturation of human fetal pancreas in diabetic nude mouse recipients, including one case of reversal of diabetes, has also been demonstrated[41]. Graft function at 52 weeks post-transplantation in the normoglycemic animal was confirmed by recurrence of diabetes on graft removal. The relative paucity of reports on pancreas transplantation in the diabetic nude rodent, probably reflects the poor survival of such animals not only in their normal immunologically incompetent state but also after induction of diabetes.

More recently, studies have shown that isolated human islets can successfully reverse diabetes in the nude rodent. RICORDI et al.[31, 33] utilized the nude mouse, made diabetic with streptozotocin, and demonstrated long-term reversal of the blood glucose following transplantation of fresh, cultured and cryopreserved human islets.

In addition, LAKE et al.[18, 19] using the nude rat and a specifically developed model with a very short (2-day) diabetic induction period, were able to rapidly reverse the diabetic state with 2-day cultured human islets. Furthermore, the engrafted islets in the normoglycemic recipients responded appropriately to a glucose challenge. The failure of aliquots of islets from a particular pancreas to reverse the diabetic state was of interest and both research groups believe that the diabetic nude rodent model offers a relatively simple method of testing the *in vivo* functional state of human islet preparations.

SUMMARY

Recent progress in islet isolation from the pancreas of large mammals including man, accentuated the need for the development of precise and reproducible techniques to assess islet yield. In this report both quantitative and qualitative criteria for islet isolation assessment were discussed, the main topics being the determination of number, volume, purity, morphologic integrity and *in vitro* and *in vivo* function tests of the final islet preparations. It has been recommended that dithizone should be used as a specific stain for immediate detection of islet tissue making it possible to estimate both the total number of islets (dividing them into classes of 50 μ diameter range increments) and the purity of the final preparation. Appropriate morphological assessment should include confirmation of islet identification, assessment of the morphological integrity and of the purity of the islet preparation. The use of fluorometric inclusion and exclusion dyes together have been suggested as a viability assay to simultaneously quantitate the proportion of cells that are intact or damaged. Perifusion of islets with glucose provides a dynamic profile of glucose-mediated insulin release and of the ability of the cells to down regulate insulin secretion after the glycemic challenge is interrupted. Although perifusion data provides a useful guide to islet viability the quantity and kinetics of insulin release do not necessarily predict islet performance after implantation. Therefore, the ultimate test of islet viability is their function after transplantation into a diabetic recipient. For this reason, *in vivo* models of transplantation of an aliquot of the final islet preparation into diabetic nude (athymic) rodents have been suggested. We hope that these general guidelines will be of assistance to standardize the assessment of islet isolations, making it possible to better interpret and compare procedures from different centers.

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