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Rapid Selection of Viable Transplantable Human Fetal Pancreatic Islets by Trypan Blue Exclusion (42335)

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Abstract. A rapid method of separating viable from nonviable human fetal pancreatic islets prior to transplantation is needed to quantify grafted tissue and optimize the possibility of successful treatment of diabetes mellitus in the recipient. After incubation with 0.04% trypan blue in isotonic Krebs–Ringer buffer solution for 15 min, the percentage of islets that excluded trypan blue was found to correlate well with fractional stimulated insulin secretion rates. The incubation procedure did not alter the subsequent insulin secretory capacity of the islets. Trypan blue exclusion rapidly and reliably identifies viable functional islet tissue prior to transplantation.

In mice the amount of fetal pancreatic islets transplanted into a diabetic recipient is a major factor in determining the reversal of hyperglycemia (1, 2). The effect of ischemia at varying temperatures on the survival of human fetal endocrine cells is a constant problem prior to transplantation. An additional factor is the autolysis produced in the pancreas during storage by activation of exocrine secretions which *in vitro* may aggravate damage.

Trypan blue is a common vital dye utilized in many laboratories as a convenient method for assessing cell viability in suspensions, e.g., leukocytes and ascites cells (3). The experiments reported here were performed to test the efficacy of using trypan blue to differentiate damaged or dead human fetal islets from viable functional islets without damaging the islets. Trypan blue was found to have these qualities plus the ease of visualization. The ratios of unstained viable functional to stained dead human fetal islets were quantitatively correlated to data of fractional stimulated insulin secretion rates obtained from fetal islets exposed to secretagogues in static *in vitro* experiments.

Our results demonstrate the usefulness of trypan blue in selecting variable functional human fetal islets from cultured suspensions before transplantation into diabetic recipients.

Materials and Methods. *Subjects.* The human fetal pancreata used in this study were obtained from the National Diabetes Research Interchange (Philadelphia, Pa.) following dilation and extraction of living fetuses. Gestational ages ranged from 16.5 to 21 weeks.

Human fetal islet preparation. Pancreata attached to the surrounding tissue were aseptically removed and stored in ice-cold sterile RPMI 1640 culture medium (GIBCO) containing 20 m*M* Hepes and 10% FCS, pH 7.4, from 60 to 144 hr. After various periods of cold storage, pancreata were removed from the surrounding tissue, chopped into fine fragments, and digested in warm (38°C) magnesium-free Hanks' buffered salt solution (HBSS) containing 7 mg/ml collagenase (Sigma grade V), 5 m*M* glucose, and 1% human albumin for 75 min in an agitator water bath (shaking 120/min). The digest was washed three times with ice-cold HBSS and samples of islets were selected under a microscope in sizes from 100 to 300 μm, and transferred to nontissue petri dishes and incubated for 48 hr at 37°C in 95% air/5% CO_2 with RPMI 1640 supplemented with 10% human adult serum. Each petri dish contained islets isolated from a single fetal donor pancreas. The number of islets isolated from each pancreas usually was of the order 800–1500.

Staining of cultured human fetal islets. Fetal islets were incubated in a solution of trypan blue containing 0.04% of the stain in isotonic Krebs–Ringer buffer (KRB) for 15 min, carefully washed several times in KRB, and islets were counted under a microscope by an observer unaware of the results of the insulin assay. The percentage of unstained fetal islets was calculated.

Static in vitro experiments. Glucose-stimulated responses for insulin release in two successive 1-hr static incubations were performed

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as previously described (4). Briefly, after culture and staining, islets isolated from each fetal pancreas were transferred to plastic tubes and incubated in KRB, pH 7.40, containing 25 m*M* Hepes, 2 m*M* glucose, and 0.3% BSA at 37°C in an atmosphere of 95% O_2/5% CO_2. After a first period of 1 hr to stabilize basal secretion, buffer was removed and a fresh KRB solution identical to the above but containing sufficient glucose to bring the total concentration to 25 m*M*, plus 1 m*M* 3-isobutyl-1-methyl-xanthine (IBMX) as potentiator, was introduced and islets were reincubated for an additional hour. After each incubation, samples of the buffer were removed for radioimmunoassay of insulin released using human insulin as reference standard. Insulin remaining within the islets at the end of the second static incubation was extracted with acidic ethanol and assayed. The fractional stimulated insulin secretion rate is defined as the amount of insulin released during the second hour of incubation and expressed as a percentage of total insulin content taken as the sum of the insulin secreted during the second hour plus the final nonsecreted insulin content (i.e., stained plus unstained islets) at the end of the experiment. Hence, the fractional stimulated insulin secretion rate quantitates the viability of a given fetal islet preparation.

Statistical analysis. The coefficient of correlation (r) and the level of significance (P) were calculated using an appropriate computer program.

Results. Only pancreatic islets could be observed under miroscopic examination. The unstained islets appear intact with no signs of morphologic changes indicating the apparent nontoxic nature of trypan blue during short-term incubation with the dye. Stained fetal islets were easily separated under a microscope from the unstained islets using a modified Pasteur pipet.

The results in Fig. 1 show experiments from eight individual fetuses. The pancreatic islets were isolated from fetal tissue after cold storage at 0–2°C for periods of 60–144 hr. The isolated fetal islets were cultured for 48 hr before static viability assays and staining were performed. A linear relationship correlated the percentage of unstained viable functional islets with their fractional stimulated insulin secretion rates (r = 0.94, $P < 0.001$). The data also indicate that stained fetal islets do not secrete insulin during maximal stimulatory conditions, i.e., high glucose concentration plus potentiator.

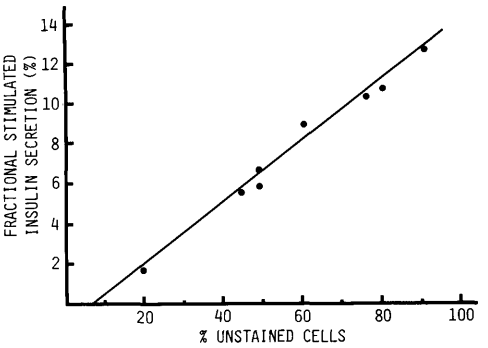

FIG. 1 Correlation between percentages of unstained viable functional human fetal pancreatic islets and their fractional stimulated insulin secretion rates. Pancreatic islets were isolated from human fetal pancreata cold stored at 0–2°C for periods of 60–144 hr. Gestational ages ranged from 16.5 to 21 weeks. Isolated islets were cultured for 48 hr before static viability assays and trypan blue staining were performed. $r = 0.94$, $P < 0.001$.

Discussion. During transportation from centers widely separated from the site of transplantation human fetal tissue has been exposed to cold ischemia, and the use of trypan blue exclusion to rapidly distinguish viable islets from damaged islets has not been reported before. The present results demonstrate that exposure to trypan blue is a rapid technique for the selection of unstained viable functional human fetal pancreatic islets from stained islets in which the permeability of the plasma membrane has been changed. Furthermore, our data show a direct proportionality between two quantitative viability tests, i.e., the percentage of unstained fetal islets in a given preparation to the fractional stimulated insulin secretion rate. Although our data do not provide evidence indicating that trypan blue stained fetal islets are metabolically dead it does show that the function of the stimulus–secretion coupling required for the release of insulin is either impaired or has ceased. Thus the inactivity of the energy requiring insulin secretory machinery might be a sign of both damage of the integrity of the islet β-cell plasma membrane and metabolic death in-

cluding arrest of the energy producing oxidative metabolism during the various cold storage periods (5–7).

We cannot exclude the possibility of a delayed effect of trace amounts of trypan blue taken up by visually unstained fetal islets. Trypan blue has been recognized as a mutagen as well as a carcinogen (8, 9) in experimental animals, but recent studies have indicated that up to 1 year of chronic protracted exposure to trypan blue was required to induce, e.g., reticuloendothelial neoplasm, predominantly in the liver and lymphomas (10, 11). Therefore, we expect that 15 min *in vitro* exposure of the human fetal islets to trypan blue would have negligible physiological effects on viable functional fetal islets and would not place the recipient of tissue treated in this manner at risk.

Our data, therefore, suggest that the *in vitro* trypan blue staining technique, in comparison to the time consuming assay of fractional stimulated insulin secretion rates, provides a rapid nondestructive means of analysis of the viability of human fetal islets for transplantation and may be used as a satisfactory way of assessing their potential in providing a functional graft.

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