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DEVELOPMENT IN VITRO OF EPITHELIAL-CELL MONOLAYERS DERIVED FROM FETAL RAT PANCREAS

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SUMMARY

Purified epithelial-cell monolayers were generated in vitro from explants of fetal rat pancreas. The extent of the development of the epithelial monolayer, as determined by planimetric analysis, was enhanced by the application of two methodological procedures: (a) preincubation of fetal pancreas in situ at 27° C for 5 hr prior to dissection and explantation; and (b) incubation of the explants in medium containing a high concentration (50% to 70%) of fetal bovine serum. By utilizing such culture conditions, sheets of contiguous epithelial cells, with little or no peripheral fibroblastic contamination, were maintained for 9 days. Whereas the majority of cells within the monolayer had morphological characteristics of pancreatic ductal cells, endocrine cells were identified by the specific immunocytochemical localization of insulin and glucagon. In addition, insulin could be detected in the incubation medium throughout the course of the experiment. The simplicity of this preparation offers some advantages over other techniques including reduced chance of contamination and reduced cellular damage or death. It provides a model for future studies directed toward developing individual cell strains derived from pancreatic epithelial cells.

Key words: fetal rat; pancreatic epithelium; cell culture; fibroblasts.

INTRODUCTION

Monolayers have been initiated in vitro from enzymatically dissociated pancreas in attempts to develop purified preparations of mammalian islet endocrine cells (1–4). In most instances, however, endocrine cells constituted a small minority of the cells present in the cultures (5, 6). Extensive contamination by fibroblasts has been a major problem (1–6). These cells proliferate rapidly. The fibroblast overgrowth limits epithelial-cell development, and interferes with physiological studies involving the endocrine cells. A number of techniques have been applied to primary cell cultures of the pancreas in an attempt to eliminate this problem (2–4, 6–8).

The purpose of the present study was to develop a methodology for the establishment of epithelial-cell monolayers derived from fetal rat pancreatic explants. Explants rather than dissociated tissue were used to generate the primary cell cultures. Cellular damage was reduced since the tissue was not subjected to harsh enzymatic treatment or mechanical agitation. This permitted the use of small amounts of tissue to generate the primary cultures. In addition to endocrine cells, other populations of pancreatic epithelial cells (e.g. duct cells) were maintained for study. Fetal pancreas was chosen because our previous experience with this tissue in organ culture indicated that both the islet- and duct-cell components continued to grow and differentiate in vitro (9). Despite this apparent advantage, its investigation in monolayer culture has been limited (1, 4, 10, 11). Additionally, a preparation of epithelial cells from a fetal source could provide a superior model for future studies concerned with endocrine-cell differentiation.

MATERIALS AND METHODS

Animals. Fetal rats (20 days postcoitum) of the Sprague-Dawley strain (Bio-Labs) were used. Fetal age was determined from witnessed matings. Pregnant females were anesthetized with ether and decapitated. Sterile dissection instruments were used to incise the shaved abdominal wall. The uterus was removed to a sterile petri dish, and fetuses were aseptically freed from uterine, amniotic and placental tissues. Within an individual experiment, fetuses were always obtained from the same adult donor.

Tissue culture. For routine culture, explants were prepared from fetuses immediately following decapitation of the mother. The pancreas was removed from each fetus and placed in sterile Tyrode's solution (pH 7.3). The pancreas was dissected free, and trimmed of surrounding connective tissue and cut into 70 explants of approximately equal dimensions (1/4-mm^3). Explants from one pancreas were transferred to a single chamber (Lab-Tek 4801) glass tissue-culture slide, and 3.0 ml of culture medium was added. Cultures were maintained in medium 199 with Hanks' salts supplemented with 10% (v/v) fetal bovine serum (FBS) (GIBCO, lot A044320; or Armour, lot N50605).

For preincubation studies, the gravid uterus was removed immediately following decapitation of the mother. Individual fetuses then were removed from the uterus, dissected free of the amniotic membrane, placed in a sterile petri dish and incubated at 27° C. Pancreatic explants were prepared from these fetuses at hourly intervals (1 to 8 hr, 27° C). Dissection was identical to that previously described. The culture medium consisted of medium 199 plus 70% FBS (Armour, lot N50605).

In experiments to test the effects of serum concentration, explants were prepared from fetuses that had been preincubated at 27° C for 5 hr. The culture media consisted of various concentrations of FBS (20% to 100%) with medium 199. Both lots of FBS from the two different commercial suppliers were used. In subsequent experiments, the suitability of several lots of FBS was qualitatively determined.

Cultures were incubated at 36° C in an atmosphere of 95% O_2/5% CO_2 which was renewed daily. No antibiotics were added to the culture media. Explants were maintained in vitro for 7 or 9 days, and the culture media were changed on days 4 and 7.

Histological preparation of tissue. Culture chamber slides with attached pancreatic tissue were rinsed twice with medium 199 to remove serum and cellular debris. The wall portion of each culture unit was removed and the glass slide with attached tissue was fixed in Bouin's solution for 1 hr. The remaining plastic adhesive then was removed. Slides were stained routinely with aldehyde fuchsin (12) and counterstained with Ponceau de xylidine or with Richardson's stain (13). Cellular localization of insulin and glucagon was accomplished using a modification (14) of the unlabeled-antibody enzyme (peroxidase) technique (15). The primary antisera (guinea-pig anti-insulin and rabbit anti-glucagon) were generated in the department and have been fully characterized (16, 17). Tissues to be sectioned were fixed in Zenker-Formalin (preincubated and nonpreincubated pancreas), embedded in paraffin, cut at 4 μm and stained with hematoxylin and eosin.

Morphometry. Stained slides were examined with a projection microscope. Epithelial cells were defined as contiguous polygonal-shaped cells that developed as cell sheets. Fibroblasts were defined as noncontiguous stellate- or spindle-shaped cells that did not develop as cell sheets. The residual explant was that area of the original explant remaining during the development of the monolayer. Tissue images were projected at a fixed magnification (×35) on table paper and traced. Areas of individual pancreatic epithelial monolayers (residual explant and peripheral epithelial cells) and/or peripheral fibroblasts were quantitated by planimetry. Projected areas were corrected for magnification. Statistical analysis was completed utilizing Student's *t*-test with a two-tailed test statistic.

Biochemical analysis. Insulin levels in the culture media were determined by radioimmunoassay (18). Insulin activity (mU per culture), was determined against rat insulin standards (Novo 25 U per mg). Amylase activity in the culture and dissection media was determined (19). Amylase content (μg per culture or μg per ml) was determined against hog pancreatic α-amylase standards (Sigma A-6255, 750 U per mg).

RESULTS

Explants grown by standard culture methodology (immediate dissection and explantation of pancreas into medium 199 supplemented with 10% FBS) failed to give rise to epithelial monolayers during 7 days in vitro. Instead, an extensive fibroblast monolayer developed (Fig. 1*A*).

Effects of preincubation on epithelial-cell development in vitro. Preincubation of fetal pancreas in vivo at 27° C prior to culture enhanced the development of epithelial-cell monolayers from explants grown in medium containing an optimal FBS level (Fig. 1*B*). Following 7 days in vitro, planimetric analysis of the 20 largest monolayers from each culture indicated that maximal epithelial monolayer area developed from pancreas preincubated for 5 hr (Table 1). Mean epithelial monolayer area in one series of experiments increased from 0.44±0.04 mm^2 at 1-hr preincuba-

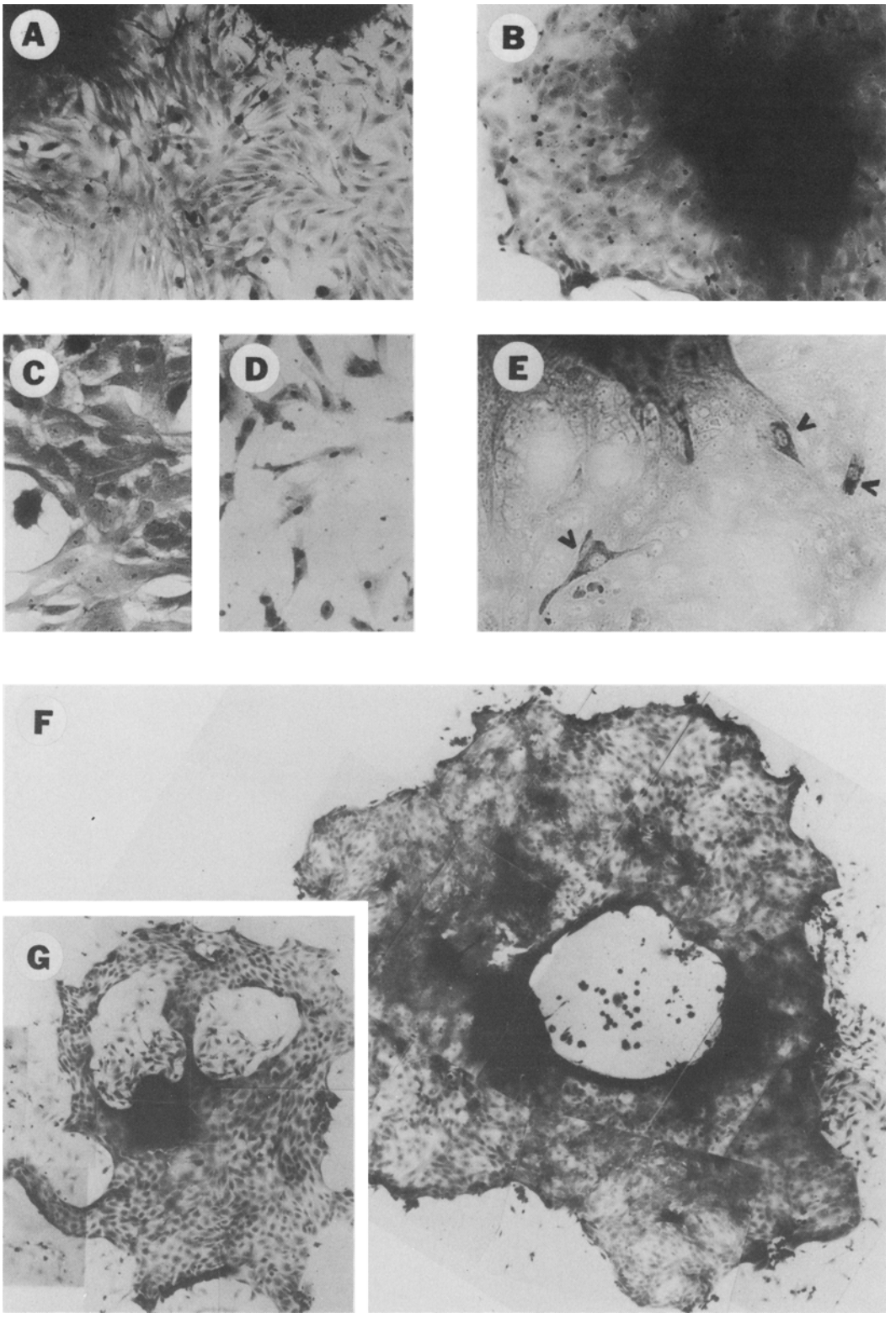
A
B
C
D
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TABLE 1

EFFECT OF PREINCUBATION OF FETAL PANCREATIC EXPLANTS ON CELLULAR MONOLAYER AREA DEVELOPMENT AFTER 7 DAYS IN VITRO

Exp. No.	Monolayer Component	Hours of Preincubation at 27° C Prior to Explantation							
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
1	Epithelial cell area[a] ($mm^2 \times 10^{-2}$)	44±4[b]	50±5	41±2	91±7	101±4	84±5	62±5	69±5
	Fibroblast area ($mm^2 \times 10^{-2}$)	220±70	140±30	180±40	60±30	21±10	30±10	19±5	20±5
2	Epithelial cell area[a] ($mm^2 \times 10^{-2}$)	39±3	57±3	80±4	77±6	89±3	79±5		
	Fibroblast area ($mm^2 \times 10^{-2}$)	362±22	160±50	62±30	40±22	24±10	10±5		

[a] Epithelial cell area includes contiguous cells and residual explant.
[b] Mean±S.E.M.

tion to a maximum of 1.01±0.04 at 5 hr of preincubation (130% increase; $p<0.001$). A similar 128% increase in epithelial monolayer area was observed in a second series of experiments. The enhanced surface area was due to the development of the true peripheral monolayer portion since the explant portion progressively decreased to small residual mass.

Effects of preincubation on in vivo fetal pancreas. Histological examination of pancreas fixed after 5 hr of preincubation revealed dissociated groups of acini. Most acinar cells appeared to be intact and contained numerous eosinophilic zymogen granules. Analysis of the dissection medium in which the explants were prepared showed only a slight increase in the level of amylase from a mean of 1.6 μg per ml with 1 hr of preincubation of the pancreas to a mean of 2.7 μg per ml with 6 hr of preincubation.

Effects of preincubation on fibroblast development in vitro. Extended preincubation of fetal pancreas prior to explant preparation and culture was deleterious to the development of fibroblasts in the monolayer (Table 1). The absolute area of peripheral fibroblasts surrounding an epithelial monolayer after 7 days in culture dropped by 93% from 3.62±0.22 mm^2 with 1 hr of preincubation to 0.24±0.10 mm^2 with 5 hr of preincubation. A similar decrease of 90% was observed in a second series of experiments ($p<0.001$). When expressed as the percent of total monolayer, the fibroblast contamination area decreased by 76% to 81% with 5 hr of preincubation ($p<0.001$).

Effects of fetal bovine serum on epithelial-cell development. High concentrations of FBS augmented the development of epithelial monolayers from preincubated (5 hr) pancreatic explants (Table 2). After 7 days in culture, planimetric analysis of the 20 largest monolayers from each culture indicated that monolayers of maximum area were produced in medium containing 50% to 70% FBS. Monolayer area increased by 31% [from 0.48±0.02 mm^2 at 20% FBS to 0.63±0.03 mm^2 in cultures grown with 50% FBS ($p<0.001$)]. Further increases were not induced by raising FBS concentration. With a second lot of FBS,

FIG. 1. *A*, Immediate explantation (nonpreincubation) of fetal pancreas in medium containing 10% FBS. After 7 days, the explants were surrounded by a network of fibroblasts in monolayer. Richardson's stain. ×90. *B*, Delay of explantation for 5 hr (preincubation) at 27° C and subsequent culture in medium containing 50% to 70% FBS almost entirely eliminated fibroblasts. After 7 days, the residual explant was surrounded by contiguous epithelial monolayer. Richardson's stain. ×90. *C*, Fibroblasts after 4 days in medium containing 20% FBS (lot A044320). Cells showed no cytotoxic effects. Richardson's stain. ×265. *D*, Necrosis of fibroblasts after 4 days in high-serum medium (50%, lot A044320). Cells contained pyknotic nuclei and had shrunken cytoplasm. Richardson's stain. ×265. *E*, Islet β-cells containing insulin-positive granules were still present within the epithelial monolayer after 9 days in vitro (*arrows*). Insulin immunocytochemical localization. ×265. *F*, Following 9 days in vitro, under optimum conditions, epithelial monolayers were polymorphous. Spaces were frequently present within cellular sheet. Richardson's stain. ×49. *G*, Following 7 days in vitro, under optimum conditions, monolayers were polymorphous and consisted of contiguous epithelial cells with few contaminating fibroblasts. Richardson's stain. ×49.

TABLE 2

EFFECT OF MEDIUM FETAL BOVINE SERUM CONCENTRATIONS ON CELLULAR MONOLAYER AREA DEVELOPMENT FROM FETAL PANCREATIC EXPLANTS AFTER 7 DAYS IN VITRO

Exp. No.	Monolayer Component	Medium % Fetal Bovine Serum								
		20	*30*	*40*	*50*	*60*	*70*	*80*	*90*	*100*
1 Lot No. A044320	Epithelial cell area[a] ($mm^2 \times 10^{-2}$)	48±2[b]	55±3	53±4	63±3	65±3	67±5			
	Fibroblast area ($mm^2 \times 10^{-2}$)	263±38	276±40	256±24	17±10	6±2	8±2			
2 Lot No. N50605	Epithelial cell area[a] ($mm^2 \times 10^{-2}$)				68±4	68±5	67±5	67±4	50±4	43±3
	Fibroblast area ($mm^2 \times 10^{-2}$)				196±20	229±29	23±12	12±8	2±2	2±2

[a] Epithelial cell area includes contiguous cells and residual explant.
[b] Mean ± SEM.

maximum monolayer area was observed at FBS concentrations of 50% to 80%. Raising the medium FBS to 100% resulted in a decline in monolayer area (from 0.67±0.04 mm^2 at 80% FBS to 0.43±0.03 mm^2 at 100% FBS). At FBS levels in which the two experiments overlapped (50% to 70%), no difference in monolayer development was noted between the two lots.

Effects of FBS on fibroblast development. FBS, when added to the culture medium at high concentrations (50% and 70%), proved to be deleterious to the development of fibroblasts (Table 2). The absolute area of peripheral fibroblasts per epithelial monolayer after 7 days in culture was 2.63±0.38 mm^2 at 20% FBS (lot A044320). No change in area was detected at 30% and 40% FBS. However, at 50% FBS a 93% decrease in fibroblast area (to 0.17±0.10 mm^2, $p<0.001$) was observed. At higher FBS concentration, further decreases were not observed. Similar results were obtained in another series of experiments with a different lot of serum; however, a 70% FBS (lot N50605) medium concentration was required before a 90% reduction in fibroblast area was observed. The absolute area of peripheral fibroblasts dropped from 2.29±0.29 mm^2 at 60% FBS to 0.23±0.12 mm^2 at 70% FBS ($p<0.001$). Incubation in 90% and 100% FBS decreased the area of fibroblasts to near zero. Expressed as the percent of total monolayer area, the fibroblast contamination of the epithelial monolayers decreased by 86% ($p<0.001$) when the FBS concentration was raised to 50% to 70%.

Histological examination of cultures maintained for 4 days in a high concentration of FBS (50%, lot A044320) revealed necrotic fibroblastoid cells. Most were shrunken and contained pyknotic nuclei (Fig. 1*D*). In contrast, control cultures maintained in low-serum medium (20% FBS, lot A044320) more optimal to the growth of fibroblasts exhibited no cytotoxic effects (Fig. 1*C*).

Variation in serum lots. Of 10 additional lots of FBS tested for culturing fetal pancreas in monolayer, only three were found acceptable. Each promoted maximal epithelial monolayer development at a specific medium level (from 50% to 70%). Compared with the previously quantitated lots A044320 and N50605, fibroblast contamination appeared to be as low or lower. Unacceptable lots showed large variation in the capacity to promote the development of epithelial monolayers and to reduce fibroblasts. Some neither promoted monolayers nor reduced fibroblasts, and one lot was decidedly toxic.

Histology of epithelial monolayers (5-hr preincubated fetal pancreas grown with 50% to 70% FBS). After 7 days in vitro, monolayers consisted of contiguous epithelial cells (Fig. 1*G*). Differentiated acinar cells with characteristic eosinophilic zymogen granulation were not identified (medium amylase was 7.2±0.3, 0.9±0.3 and <0.5 μg per culture during days 0 to 4, 4 to 7 and 7 to 9, respectively). Glucagon-positive α_2-cells were present in the monolayer. Insulin-positive islet β-cells (Fig. 1*E*) were also present in groups or scattered singly throughout the monolayers (medium insulin was 1.24±0.04, 1.28±0.11 and 0.91±0.08 mU per culture during culture days 0 to 4, 4 to 7 and 7 to 9 respectively). Nine-day monolayers revealed a similar morphology except that monolayer area had increased (Fig. 1*F*). Histological

examination of the residual explant portion of the developing monolayers (5 days in vitro) revealed many surviving pancreatic ducts.

DISCUSSION

Primary cultures of fetal rat pancreatic explants gave rise to extensive fibroblast monolayers following 1 week in vitro. In contrast, if the pancreas was preincubated in situ at room temperature (27° C) for an extended period (about 5 hr) prior to culture, epithelial monolayer development was promoted and fibroblast contamination reduced. Long periods of cold ischemia (4° C) are compatible with the survival of both islet and ductal cells in organ culture of human fetal pancreas (20). Ferguson et al. (21) reported that islets stored in a balanced salt solution at 4° C for 15 hr retained 100% histological and functional survival. However, the mechanism by which the preincubation period promotes epithelial-cell monolayers is not clear. Histological examination of fetal pancreas at the end of optimal preincubation revealed a disruption of the stroma. However, nearly all of the acinar cells appeared intact and packed with zymogen granules.

Jones and Trump (22), using ischemic rat pancreatic slices incubated at 37° C, also observed no major change in acinar cell morphology (light and electron microscopy) until 8 hr of incubation. Thus, morphologically, it appeared that autodigestion of stromal elements by the exocrine pancreas contributed little to the effect of preincubation. Further analysis of the dissection medium in which explants were prepared showed only a small increase in amylase activity as preincubation was increased from 1 to 6 hr. The result again suggests that few acinar cells undergo autolysis during preincubation.

A high medium level of fetal bovine serum (50% to 80%) augmented maximal epithelial monolayer development from preincubated pancreatic explants. A number of factors promoting cell proliferation (nonsuppressible insulin-like activity, multiplication activity, the somatomedins, insulin, etc.) are present in sera (23–27). In addition, recent studies have demonstrated that the development of epithelial cells from the explants as contiguous sheets (on glass and plastic substrates) resulted primarily from the locomotory activity of the sheet's marginal cells (28). Serum was a necessary medium component to permit epithelial cell migration (29). Other investigators have demonstrated a serum factor that promotes fibroblast migration without DNA synthesis (30). More recently, serum has been reported to contain a factor (or factors) that induces cell attachment and spreading on a substrate—conditions necessary for cell migration (24, 31). High medium levels of FBS (certain lots) may have provided a more optimal level of these factors and thus augmented monolayer development by stimulating cell proliferation or by increasing the ability of fetal pancreatic epithelial cells to migrate on the glass substrate.

The mechanism by which certain lots of FBS (50% and 70% medium levels) induced the selective necrosis of fibroblasts is unknown at present. Necrosis related to variation in the medium concentration of essential metabolites cannot be excluded by the present experiment. A similar fibroblast necrosis in neonatal rat pancreatic monolayers has been reported when the cells were cultured in the presence of 100% FBS (8). Substitution of cystine-free medium for 100% FBS duplicated the effect. The negative effects of endogenous steroid hormones in FBS on the plating and cloning efficiency of human fibroblasts have been reported (32). Lots of FBS high in progesterone did not support growth. Sera with high cloning efficiencies could be made nonsupportive by the addition of progesterone. Thus the variability of FBS lots to promote the development of epithelial monolayers and reduce fibroblasts may be related to variability found in the steroid concentration (33).

The epithelial monolayers were composed primarily of contiguous cells lacking specific staining or organizational characteristics of either islet endocrine or acinar cells. These epithelial cells are probably derived from pancreatic ductal cells since histological examination of sections of the residual explants revealed them to be comprised primarily of ductal elements. It is also possible that some of the epithelial monolayers may represent dedifferentiated acinar cells. Evidence suggesting this possibility in pancreatic monolayer cultures has been reported (34, 35). Medium amylase activity fell quickly and could not be detected in the medium during culture days 7 to 9 which correlated with the lack of identifiable acinar cells in the culture. In contrast, islet β-cells were identifiable in the epithelial-cell sheets and continued to secrete insulin throughout the entire culture period. Glucagon-positive α_2-cells were also present. Thus the explant method promotes the survival of both presumptive duct cells and islet β- and α_2-cells, whereas differentiated acinar cells are not

maintained. Quantitative studies are in progress to assess the role of culture conditions on the development of the endocrine cells in vitro.

A comparison of the results obtained with the present method utilizing fetal rat pancreatic tissue with the previously reported method involving enzymatic dissociation of neonatal and adult rat pancreas (2, 3, 10, 34, 36) indicates a number of similarities, including the selective loss of acinar tissue and medium amylase activity and the maintenance of islet tissue. However, a number of differences are apparent. The explant method promotes not only the survival of islet endocrine cells, but also the survival of undifferentiated epithelial cells (presumptive duct cells) as the major cell type. This offers the possibility of future studies concerned with the factors influencing the differentiation of pancreatic duct cells into endocrine and/or exocrine cells. The explant eliminates the need for the multistep dissociation process and further permits the use of small amounts of starting tissue to establish a primary culture. In addition, the current method reduces considerably the number of contaminating fibroblasts, thus raising the percentage of epithelial cells in the culture and maximizing the chances of establishing cell strains derived from specific pancreatic epithelial cell types.

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