# MONOLAYER CULTURES DERIVED FROM NEONATAL HAMSTER PANCREAS

## Light and Electron Microscopy

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ABSTRACT

Cells derived by trypsinization of neonatal golden hamster pancreas were cultured in modified Eagle's medium for 120 h in the presence of glucose (0.8 mg/ml) and for an additional 48 h in medium containing glucose (0.8 or 3.1 mg/ml) or tolbutamide (1,000 μg/ml) plus glucose (0.8 mg/ml). At day 7, cultures were stained differentially for light microscopy or examined by electron microscopy. Immunoreactive insulin (IRI) and immunoreactive glucagon (IRG) in the culture medium were measured by standard immunoassay procedures.

Staining properties and ultrastructural appearance of cultured cells were comparable to those of the intact neonatal hamster pancreas. Cultures consisted predominantly of cells possessing aldehyde fuchsin positive ($AF^+$) cytoplasmic granules resembling ultrastructurally those of the intact neonatal pancreatic beta cells and additionally, those of fibroblastoid, acinar, acino-insular, and aldehyde fuchsin negative ($AF^-$) argyrophilic cells. IRI release rate by the cultured cells was increased in the presence of elevated glucose or tolbutamide which paralleled the loss of $AF^+$ granulation, but IRG release rate was suppressed by elevated glucose concentration.

These findings indicate that these monolayer cultures consist of most of the cell types occurring in the neonatal pancreas, including endocrinologically competent islet cells.

## INTRODUCTION

Monolayer cultures derived by enzymatic dispersion of neonatal golden hamster pancreas consist predominantly of epithelioid cells containing aldehyde fuchsin positive ($AF^+$) and insulin-immunofluorescent cytoplasmic granules, release newly synthesized immunoreactive insulin (IRI), and respond to the insulinotropic action of glucose and tolbutamide (1, 2). Although these findings provide evidence which suggests strongly that endocrinologically competent beta cells are present in these cultures, these and other constituent cells have not been identified definitively. The present report is based, therefore, on studies designed to provide more extensive cytochemical and ultrastructural characterization of golden hamster pancreatic monolayer cultures relative to the intact uncultured tissue of origin and to correlate cytostructural features with qualitative and quantitative evidence of endocrine secretory function.

## MATERIALS AND METHODS

### *Tissue Culture and Experimental Design*

Cells dispersed by trypsinization of 5-day neonatal golden hamster pancreas were cultured on glass cover slips in Leighton tubes as previously described (1). Each Leighton tube was inoculated with approximately $8 \times 10^5$ cells contained in 1 ml culture medium having a glucose concentration of 0.8 mg/ml. Culture medium was renewed at the end of the first 72 h of cultivation and at 48-h intervals thereafter subsequent to washing the attached cells three times with fresh medium to remove residual IRI. After cultivation for 120 h in the presence of glucose at 0.8 mg/ml, the cells were cultured for an additional 48 h in 1 ml fresh medium containing glucose (0.8 or 3.1 mg/ml) or sodium tolbutamide (1,000 μg/ml; Upjohn Co., Kalamazoo, Mich., lot 1209-3) plus glucose (0.8 mg/ml). Medium from individual culture tubes was analyzed directly for IRI or immunoreactive glucagon (IRG), and the corresponding cover slip preparations were examined by light or electron microscopy.

### *Light and Electron Microscopy*

A portion of the cover slip preparations from each of several cultures derived from different tissue pools were stained for light microscopy using several different procedures, each applied separately while the remainder were prepared for electron microscopy. Cover slip preparations were stained directly by the Scott aldehyde fuchsin method to demonstrate pancreatic beta granules (3); the Hellerström and Hellman or the Grimelius silver impregnation procedures to identify pancreatic alpha$_1$ or alpha$_2$ cells, respectively (4, 5); the toluidine blue stain in conjunction with aldehyde fuchsin to distinguish alpha$_1$ from beta cells (6); or the azure eosin stain to demonstrate pancreatic zymogen granules (7). Cover slip preparations used for electron microscopy were fixed in 3% gluteraldehyde in 0.20 M phosphate buffer (pH 7.2) for 1 h, rinsed two times with the buffer solution, and postfixed for 30 min in 1% $OsO_4$ in phosphate buffer at the above concentration. For each preparation, the fixed cells were scraped from the cover slip surface with a razor blade and embedded in Epon. Sections were stained with uranyl acetate followed by lead citrate and examined using an RCA EMU3G electron microscope.

For comparison, noncultured intact pancreas from 5-day neonatal golden hamster was embedded in paraffin after appropriate fixation, sectioned at 7 μm, and stained by procedures identical with those applied to the cover slip preparations or was prepared for and examined by electron microscopy basically as described above for cultured cells.

### *Immunoassay*

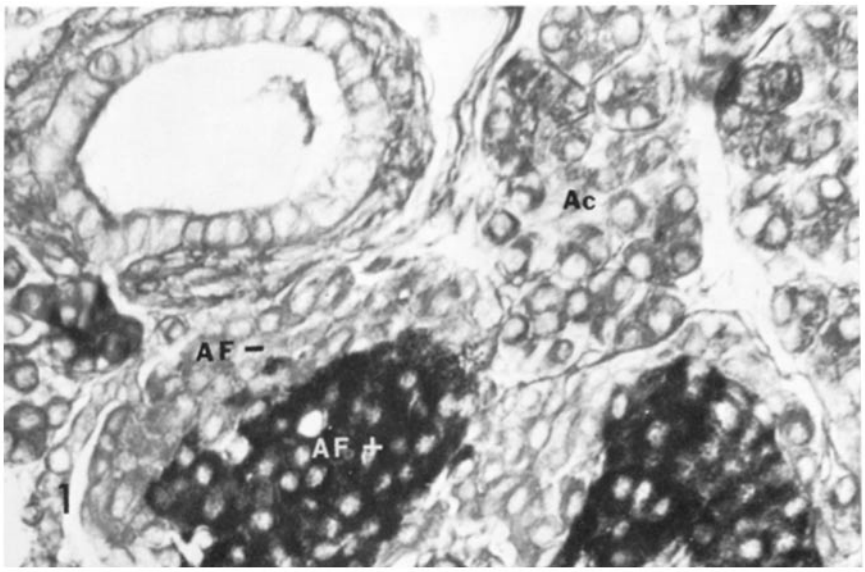
IRI was measured as described previously (1) using bovine insulin (Lilly Research Laboratories, Eli Lilly Co., Indianapolis, Ind. lot PJ4609) as a standard, while IRG was quantified by the method of Unger et al. (8) using porcine glucagon (Eli Lilly Co., lot 258234B-167-1) as a standard. For both types of assay, suitable portions of the medium from individual culture tubes were used. The data were corrected for the IRI or IRG activity in equivalent volumes of medium in which cells had not been cultured.

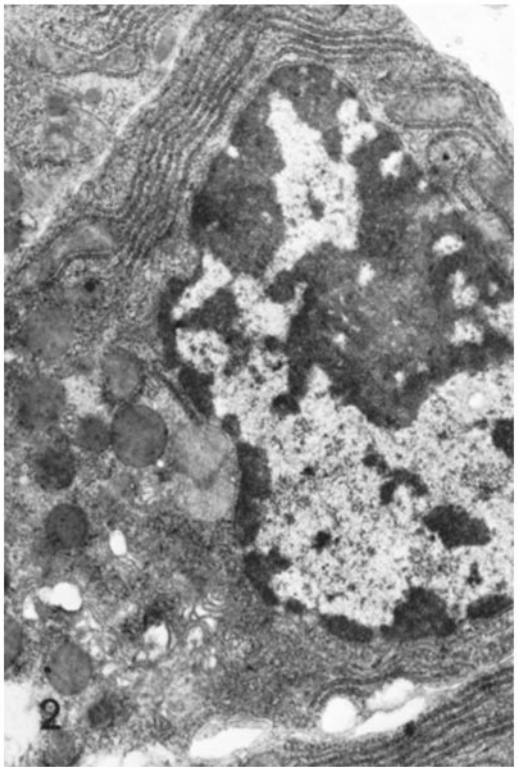
## RESULTS

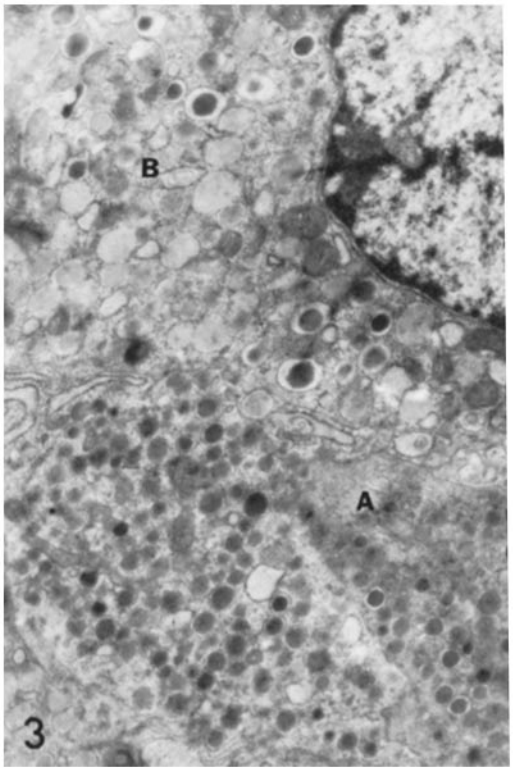
### *Intact Pancreas*

At 5 days after birth, intact hamster pancreas consisted of small islets of $AF^+$-granulated cells partially or completely separated spatially from the surrounding acinar tissue (Fig. 1). Occasionally, islets contained a peripheral halo of $AF^-$ cells not stainable by the Grimelius or the Hellerström and Hellman silver impregnation procedures or with toluidine blue. Acinar cells typically contained cytoplasmic granules which stained bright red with azure eosin.

Ultrastructurally recognizable exocrine cells were usually well differentiated (Fig. 2). The rough endoplasmaic reticulum (RER) was arranged in extensive parallel arrays; moderately electron-dense, membrane-bound, large cytoplasmic granules were numerous; mitochondria were ellipsoidal and numerous; the nucleus was irregularly shaped with unevenly dispersed chromatin and prominent nucleoli; and the Golgi complex was well developed. Granulated cells with ultrastructural characteristics of alpha or beta cells were identified also (Fig. 3). The alpha cells, when present, were sparse in number, peripherally located in the islets, and contained electron-dense granules surrounded by a tightly fitting membranous envelope. The beta cells were proportionately more numerous, centrally located in the islets, and invariably contained electron-dense granules within loosely fitting membranous sacs as well as electron-lucent granules with more closely applied membranous envelopes. In alpha and beta cells, the endoplasmic reticulum (ER) was moderately well developed, the mitochondria were ellipsoidal with cristae arranged in parallel stacks, the ribosomes were abundant, the nucleus was round to avoid with irregularly dispersed chromatin and prominent nucleoli, and the Golgi




FIGURES 1–3 Light and electron microscopy of intact 5-day neonatal golden hamster pancreas. Fig. 1. Islets showing central $AF^+$ and peripheral $AF^-$ cells surrounded by acinar tissue (*Ac*). × 1,800. Fig. 2. Exocrine pancreas showing parallel arrays of RER and moderately electron-dense granules. × 11,900. Fig. 3. Pancreatic alpha (*A*) and beta (*B*) cells, the latter containing electron-lucent as well as electron-dense cytoplasmic granules. × 11,600.

apparatus was well developed. Acino-insular cells identified by electron microscopy in pancreatic monolayer cultures (*v.i.*) were not observed in the intact pancreas.

### *Cultured Pancreas*

GENERAL MORPHOLOGY: At 7 days of cultivation, monolayer cultures derived from 5-day neonatal golden hamster pancreas consisted of varying admixtures of fibroblastoid and epithelioid cells identifiable by light microscopy (Fig. 4). Cultures were well established at this time. Cells generally were elongated and flattened, and mitotic figures were observed frequently. Two types of epithelioid cell, distinguishable from each other by distinct differences in the electron density of cytoplasmic granules, and fibroblastoid cells, characterized by the absence of granules and a paucity of other subcellular organelles, were identified by electron microscopy (Fig. 5). In the epithelioid and fibroblastoid cells, mitochondria were elongated with tubular cristae, free and membrane-bound ribosomes were abundant, and the Golgi complex generally was inconspicuous.

ACINAR CELLS: Epithelioid cells with numerous eosinophilic cytoplasmic granules were present in these cultures (Fig. 6), but they were considerably less numerous than the other constituent cell types. Ultrastructurally, these cells (Fig. 7) resembled the exocrine cells of intact 5-day neonatal pancreas. They contained moderately electron-dense, membrane-bound, large cytoplasmic granules. The RER and Golgi complex were moderately well developed, but the parallel arrays of RER characteristic of exocrine cells in the intact neonatal pancreas were not apparent in these cultured cells (cf. Fig. 2).

ALPHA CELLS: Epithelioid cells possessing argyrophilic cytoplasmic granules were detected in these cultures with the Grimelius silver staining procedure for alpha$_2$ cells (Fig. 8). These cells occurred singly or as discrete aggregates and were found infrequently. Their scarcity may well account for our failure to detect them by electron microscopy. More definitive evidence for the presence of alpha$_2$ cells in these cultures is provided, however, by the data of Table I which demonstrate the release into the culture medium of measurable IRG and suppression of its release in the presence of elevated glucose concentration. We were unable to detect alpha$_1$ cells in these cultures by the Hellerström and Hellman staining procedure.

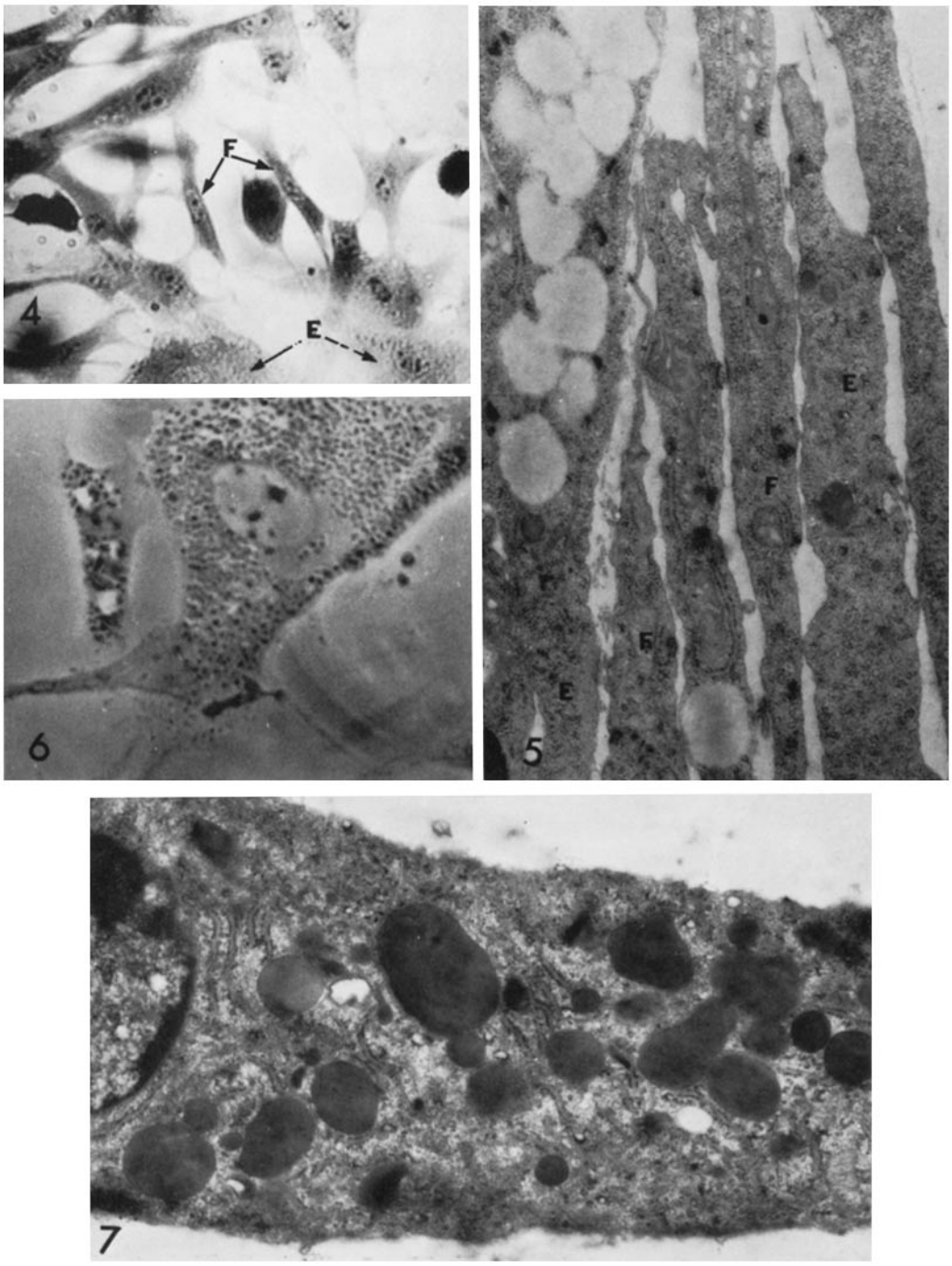
BETA CELLS: The majority of epithelioid cells in these cultures contained cytoplasmic granules, frequently concentrated in the perinuclear region, which were stained deeply by aldehyde fuchsin (Fig. 9). Ultrastructurally (Fig. 10), they resembled beta cells in the intact neonatal pancreas, except that these cultured cells contained only amorphous electron-lucent granules in contrast to the two types observed in the intact pancreas (cf. Fig. 3). Additionally, the ER in the cultured cells usually was moderately well developed and contained numerous membrane-bound ribosomes, mitochondria which were elongated with tubular cristae, and the nucleus which was ovoid with finely dispersed chromatin and distinct nucleoli.

The presence of beta cells in these cultures is suggested further by the data of Table II which demonstrate the release of IRI into the culture medium and the increase in release rate achieved in the presence of elevated glucose concentration or tolbutamide. Moreover, the increase in IRI release rate in response to elevated glucose concentration was accompanied consistently by an apparent increase in number of the AF$^+$-granulated epithelioid cell type in these cultures and concomitantly, by a degranulation of these cells evident by light and electron microscopy (Figs. 11, 12). The degree of degranulation which occurred in the presence of elevated glucose concentration was considerably greater than that in the presence of tolbutamide which was parallel with corresponding differences in stimulation of IRI release.

ACINO-INSULAR CELLS: A substantial portion of the epithelioid cells observed in these pancreatic cultures possessed exocrine and endocrine cytostructural features. These acino-insular cells (Fig. 13) contained electron-lucent granules resembling those in cultured cells identified as beta cells and electron-dense granules resembling those in the intact and cultured acinar tissue. In these acino-insular cells, mitochondria with tubular cristae were abundant, the RER was moderate in quantity, and the nucleus was ovoid with moderate amounts of chromatin and distinct nucleoli.

## DISCUSSION

Present findings demonstrate that monolayer cultures derived from neonatal golden hamster pancreas consist of a mixed population of fibro-




FIGURES 4 and 5 Cellular heterogeneity of 7-day hamster pancreatic monolayer cultures. Fig. 4. Fibroblastoid (*F*) and epithelioid (*E*) cells showing numerous mitotic figures (aldehyde fuchsin). × 640. Fig. 5. Fibroblastoid cells (*F*) and two types of epithelioid cell (*E*) distinguishable by differences in electron density of cytoplasmic granules. × 19,000.

FIGURES 6 and 7 Acinar cells in culture. Fig. 6. Cells containing numerous eosinophilic granules (azure eosin, phase contrast). × 2,000. Fig. 7. Cell containing moderately electron-dense granules. × 16,000.

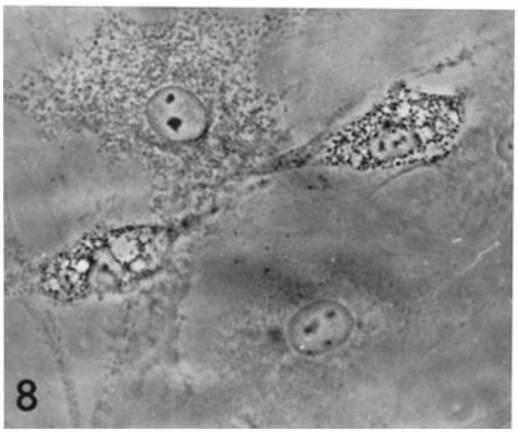



FIGURE 8 Argyrophilic cells stained by the Grimelius silver nitrate procedure (phase contrast). × 1,160.

TABLE I

*Rate of IRG Release by Hamster Pancreatic Monolayer Cultures in the Presence of Different Concentrations of Glucose*

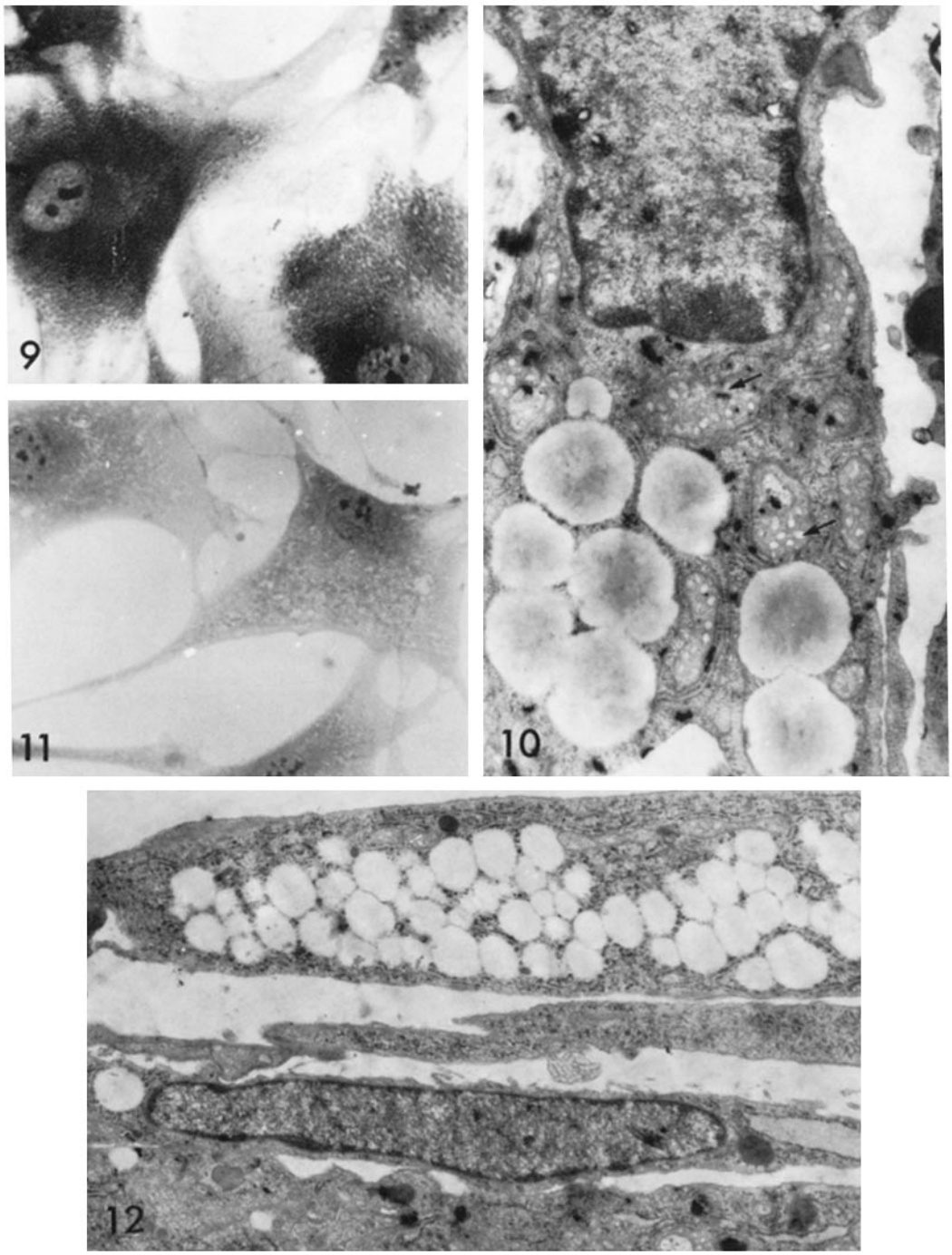
| Glucose | IRG |
|---|---|
| *mg/ml* | *pg/ml/48 h* |
| 0.8 | 320* |
| | 300‡ |
| | 280 |
| 3.1 | 220 |
| | 195 |
| | 170 |

* Values for single cover slip preparations derived from a common tissue pool for both glucose concentrations.
‡ Mean.

blastoid and epithelioid cells. The latter were identified cytochemically and/or ultrastructurally as pancreatic acinar, alpha$_2$, or beta cells. The survival in monolayer culture of most of the endocrine cell types identifiable ultrastructurally in originating pancreatic tissue also has been reported recently (9). Furthermore, present demonstration that the rate of IRI release was increased whereas that of IRG was decreased in the presence of elevated glucose concentration, provides evidence suggesting further the presence of endocrinologically competent beta and alpha$_2$ cells in these monolayer cultures. It is well known that elevated glucose concentration stimulates the secretion of insulin in a number of mammalian species, including the golden hamster (10), and evidence exists that it suppresses glucagon secretion in vivo (11) and in vitro (12, 13). Stimulation of the rate of IRI secretion and suppression of that of IRG in the presence of elevated glucose concentration also has been reported recently for monolayer cultures derived from rat pancreas (9, 14). Additional evidence suggesting the presence of endocrinologically functional beta cells in hamster pancreatic monolayer cultures is provided by the degranulation of AF$^+$-granulated cells which consistently accompanied the increase in IRI release rate observed in the presence of elevated glucose concentration or of tolbutamide. As suggested previously, degranulation of AF$^+$-granulated epithelioid cells in identical cultures is attributable, most likely, to the release of stored insulin from pancreatic beta cells (2). The possible existence of a similar relationship between alterations in the granulation of cultured alpha$_2$ cells and IRG release rate could not be established by us, however, because of the paucity of this cell type in culture.

Unlike beta cells of intact neonatal hamster pancreas which contained electron dense as well as electron-lucent cytoplasmic granules, those in monolayer culture contained the lucent granules exclusively. It has been suggested that the electron-lucent granules observed in pancreatic cells of several different mammalian species are immature secretory granules whose content has not yet condensed into the dense core normally associated with the mature beta granule (15). Our present findings suggest, therefore, that beta granule maturation is altered in culture. While the reason for this alteration is not known, it does not appear attributable to a lack of zinc in the culture medium (C. R. Scheid, and I. A. Macchi, Unpublished Observation). The development of alpha cell granulation also is suggested by our present findings. Thus, argyrophilic alpha$_2$ cells were observed only in culture despite identification by electron microscopy of cells with alpha granules in the intact neonatal pancreas. Lack of argyrophilia also observed by others in cultured fetal or neonatal rat pancreas stained by the Grimelius silver impregnation procedure has been attributed to a paucity of granules in developing alpha cells (16).

Epithelioid cells containing AF$^+$ cytoplasmic granules were the most numerous type seen by us in culture in contrast to the relative number observed in the intact neonatal pancreas. This suggests the selective survival or new formation of endocrine pancreas during cultivation. The failure




FIGURES 9–12 Epithelioid cells in culture identified as beta cells. Fig. 9. Cells showing cytoplasmic $AF^+$ granulation. × 1,280. Fig. 10. Ultrastructural appearance of the major cell type in culture showing electron-lucent granules and tubular mitochondria (arrows). × 16,400. Fig. 11. Loss of $AF^+$ granulation in the presence of elevated glucose concentration. × 1,100. Fig. 12. Ultrastructural appearance of degranulated cells in the presence of elevated glucose concentration. × 11,200.

of exocrine cells to persist in monolayer cultures derived from neonatal rat pancreas has been reported previously (9, 17, 18). New formation of endocrine tissue also has been observed previously in cultured explants of rat pancreas (19–21) and inferred to have arisen by differentiation of ductule cellular elements (20). Evidence exists, moreover, for the formation in vivo of endocrine pancreas from ductule epithelium (22). It is possible, therefore, that in the cultured hamster pancreatic monolayers, endocrinre pancreas originated from ductule cells, or, alternatively, the increased relative number of $AF^+$-granulated cells in culture may have resulted from the redifferentiation of exocrine cells. The latter explanation is suggested by the loss in the cultured exocrine cells of the parallel arrangement of RER characteristic of the intact neonatal hamster pancreas (possibly a prelude to redifferentiation) and by the occurrence in culture of numerous acino-insular cells. Orci et al. (9) recently have provided evidence suggesting dedifferentiation of exocrine cells in monolayer cultures derived from neonatal rat pancreas although these authors do not mention redifferentiation of the dedifferentiated cells. Acino-insular transformation has been observed in vivo, however, in pancreatic tissue of several other mammalian species (23–25). Thus, it appears possible that a portion of the $AF^+$-granulated cells occurring in hamster pancreatic monolayer cultures may have been derived through redifferentiation of exocrine cells. Additionally, although changes in cell density were not determined quantitatively in the present studies, elevated glucose concentration appeared to stimulate cell replication in the cultured pancreatic cells. Thus, the cell density and number of visible mitotic figures appeared to increase in cultures exposed 48 h to elevated glucose concentration. These observations are consistent with those reported by Chick et al. for monolayer cultures derived from neonatal rat pancreas (18).

TABLE II

*Rate of IRI Release by Hamster Pancreatic Monolayer Cultures in the Presence of Various Concentrations of Glucose or Tolbutamide*

| Insulinotropic agent | Concentration | IRI |
|---|---|---|
| | | *μU/ml/48 h* |
| Glucose | 0.8 mg/ml | 10 ± 1* (21 |
| | 3.1 mg/ml | 90 ± 24 (23) |
| Tolbutamide | 1,000 μg/ml | 34 ± 4 (18) |

* Mean ± SE for the number of cover slip preparations indicated in parentheses. Cover slip preparations from each of three cultures were used for each glucose concentration and for tolbutamide.

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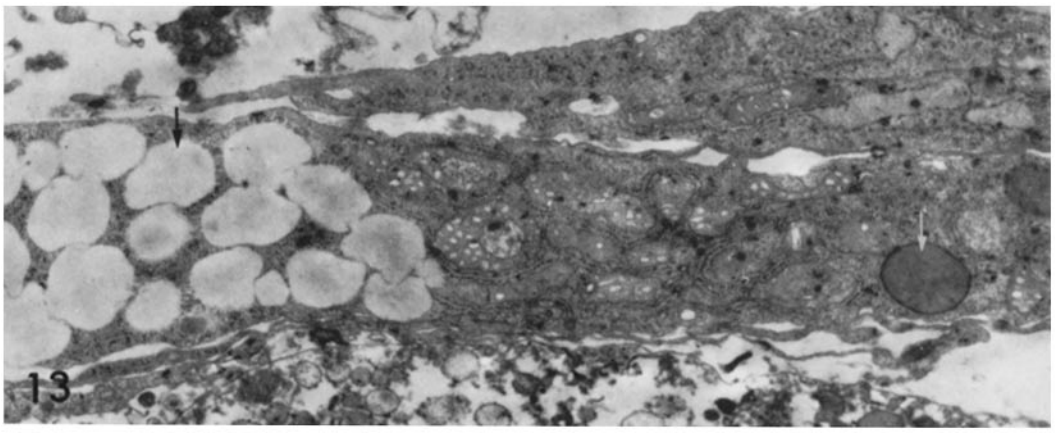


FIGURE 13 Acino-insular cell in culture showing electron-lucent granules (dark arrow) resembling pancreatic beta granules and moderately electron-dense granules (light arrow) resembling those of intact and cultured acinar tissue. × 13,700.

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## REFERENCES

1. MACCHI, I. A., and E. H. BLAUSTEIN. 1969. Cytostructure and endocrine function of monolayer cultures of neonatal hamster pancreas. *Endocrinology.* **84**:208.
2. MACCHI, I. A., W. R. BEYER, D. A. GAPP, E. H. BLAUSTEIN, and S. B. BEASER. 1973. Monolayer cultures derived from neonatal hamster pancreas: stimulation of immunoreactive insulin secretion and biosynthesis. *Proc. Soc. Exp. Biol. Med.* **143**:335.
3. SCOTT, H. R. 1952. Rapid staining of beta cell granules in pancreatic islets. *Stain Technol.* **27**:267.
4. HELLERSTRÖM, C., and B. HELLMAN. 1960. Some aspects of silver impregnation of the islets of Langerhans in the rat. *Acta Endocr.* **35**:518.
5. GRIMELIUS, L. 1968. A silver nitrate stain for $\alpha_2$ cells in human pancreatic islets. *Acta Soc. Med. Ups.* **73**:243.
6. KALLMAN, J. 1971. Aldehyde fuchsin followed by toluidine blue O for pancreatic islet cells. *Stain Technol.* **46**:211.
7. LILLIE, R. D. 1965. Histopathologic Technic and Practical Histochemistry. McGraw Hill Book Co., New York. 295.
8. UNGER, R. H., E. AGUILAR-PARADA, W. A. MÜLLER, and A. M. EISENTRAUT. 1970. Studies of pancreatic alpha cell function in normal and diabetic subjects. *J. Clin. Invest.* **49**:837.
9. ORCI, L., A. A. LIKE, M. AMHERDT, B. BLONDEL, Y. KANAZAWA, E. B. MARLISS, A. E. LAMBERT, C. B. WOLLHEIM, and A. E. RENOLD. 1973. Monolayer cell cultures of neonatal rat pancreas: an ultrastructural and biochemical study of functioning endocrine cells. *J. Ultrastruct. Res.* **43**:270.
10. SODOYEZ, J.-C., F. SODOYEZ-GOFFAUX, and P. P. FOÁ. 1969. Evidence for an insulin-induced inhibition of insulin release by isolated islets of Langerhans. *Proc. Soc. Exp. Biol. Med.* **130**:568.
11. OHNEDA, A., E. AGUILAR-PARADA, A. M. EISENTRAUT, and R. H. UNGER. 1969. Control of pancreatic glucagon secretion by glucose. *Diabetes.* **18**:1.
12. LAUBE, H., R. FUSSGÄNGER, R. GOBERNA, K. SCHRÖDER, K. STRAUB, K. SUSSMAN, and E. F. PFEIFFER. 1971. Effects of tolbutamide on insulin and glucagon secretion of the isolated perfused rat pancreas. *Horm. Metab. Res.* **3**:238.
13. IVERSON, J. 1971. Secretion of glucagon from the isolated, perfused canine pancreas. *J. Clin. Invest.* **50**:2123.
14. MARLISS, E. B., C. B. WOLHEIM, B. BLONDEL, L. ORCI, A. E. LAMBERT, W. STAUFFACHER, A. A. LIKE, and A. E. RENOLD. 1973. Insulin and glucagon release from monolayer cell cultures of pancreas from newborn rats. *Eur. J. Clin. Invest.* **3**:16.
15. WILLIAMSON, J. R., P. E. LACY, and J. W. GRISHAM. 1961. Ultrastructural changes in islets of the rat produced by tolbutamide. *Diabetes.* **10**:460.
16. SCHWEISTHAL, M. R., and C. C. FROST. 1973. Differentiation of alpha cells in the fetal rat pancreas grown in organ culture. *Am. J. Anat.* **136**:527.
17. LAMBERT, A. E., B. BLONDEL, Y. KANAZAWA, L. ORCI, and A. E. RENOLD. 1972. Monolayer cell culture of neonatal rat pancreas: light microscopy and evidence for immunoreactive insulin synthesis and release. *Endocrinology.* **90**:239.
18. CHICK, W. L., V. LAURIS, J. H. FLEWELLING, K. A. ANDREWS, and J. M. WOODRUFF. 1973. Effects of glucose on beta cells in pancreatic monolayer cultures. *Endocrinology.* **92**:212.
19. SCHWEISTHAL, M. R., L. J. WELLS, and M. P. CEAS. 1965. Development of islets and acini from the explanted primordium of the pancreas of the rat embryo. *Anat. Rec.* **151**:93.
20. MURRELL, L. R. 1966. Mammalian pancreatic islet tissue in organ culture. I. Methods of culture and in vitro histogenesis. *Exp. Cell Res.* **41**:350.
21. VECCHIO, L. R., and A. E. GONET. 1967. Culture d'organe de pancreas foetal de rat. I. Effets du glucose d'autres composants du milieu de culture et d'un sulfamide de hypoglycemiant. *Helv. Physiol. Pharmacol. Acta.* **25**:103.
22. BOQUIST, L. 1972. Fine structure of the endocrine pancreas in newborn rodents. *Diabetes.* **21**: 1051.
23. PICTET, R., L. ORCI, A. E. GONET, C. H. ROUIL-

LER, and A. E. RENOLD. 1967. Ultrastructural studies of the hyperplastic islets of Langerhans of spiny mice (*Acomys cahirinus*) before and during the development of hyperglycemia. *Diabetologia.* **3**:188.

24. LEDUC, E. H., and E. E. JONES. 1968. Acinar-islet transformation in mouse pancreas. *J. Ultrastruct. Res.* **24**:165.

25. LAMBERT, A. E., L. ORCI, and A. E. RENOLD. 1970. Some factors controlling differentiation and/or modulation of rat pancreatic islet cells. *Adv. Metab. Disord.* **1**(Suppl. 1):35.