Monolayer Cultures Derived from Neonatal Hamster Pancreas: Stimulation of Immunoreactive Insulin Secretion and Biosynthesis[1] (37315)

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The successful cultivation in monolayer of functional endocrine pancreatic cells derived by enzymatic dispersion reported for hamster pancreas (1–3) and also for several other mammalian species (4–9) suggests this as a plausible approach in deriving pure preparations of endocrinologically competent beta cells applicable to the investigation of insulin biosynthesis and the regulation of insulin secretion. Although the release of immunoreactive insulin (IRI) by hamster pancreatic monolayer cultures has been demonstrated (3), the extent to which this reflects secretion of IRI by endocrinologically competent islet cells has not been assessed. The present investigation was directed, therefore, toward demonstrating that monolayer cultures derived from neonatal golden hamster pancreas possess the ability to synthesize IRI and to respond to known insulinotropic agents.

Materials and Methods. Tissue culture and experimental procedure. Cells derived by trypsinization of 5-day neonatal golden hamster pancreas were cultured on glass coverslips in Leighton tubes at 36° employing modified Eagle's basal medium as previously described (3). Each Leighton tube was inoculated with 5×10^5 to 8×10^5 cells contained in 1 ml culture medium having a glucose concentration of 0.8 mg/ml. Cultures were not disturbed during the first 72 hr of cultivation. The medium was renewed at the end of this period and thereafter at 48 hr intervals subsequent to washing the attached cells 3× with fresh medium to remove residual IRI.

In one series of experiments, each coverslip preparation was transferred to a fresh Leighton tube on Day 3 to eliminate cells attached to the vessel wall and cultured for an additional 48 hr in fresh medium containing glucose at 0.8 mg/ml. These cultures were terminated at 5 days, and the coverslip preparations were stained by the Scott aldehyde fuchsin (AF) method (10). The AF+-granulated epithelioid cells were classified according to density of AF+ cytoplasmic granulation and counted within a uniformly representative sample area of 28 separate high-power (430×) fields comprising approximately 2% of the total coverslip surface. The total number of each AF+-granulated epithelioid cell type per coverslip was extrapolated from the 2% sample and correlated with the corresponding IRI value employing product–moment analysis (11).

In other series, cells were cultured for 120 hr in the presence of glucose at 0.8 mg/ml and then for an additional 48 hr either in 1 ml fresh leucine-deficient[3] medium to

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[3] Nonlabeled leucine content limited to that attributable to the calf serum supplement employed.

which was added L-leucine 4,5-^{3}H (0.068 μg/ml; New England Nuclear Corp., Boston, MA; sp act 54 Ci/mmole) or in 1 ml complete fresh medium containing glucose (0.8 or 3.1 mg/ml) or sodium tolbutamide (10, 100, or 1000 μg/ml; Upjohn, Lot 1209-3) plus glucose (0.8 mg/ml). Medium from individual culture tubes was analyzed directly for IRI or medium and corresponding cells recovered quantitatively were pooled separately for extraction.

Extraction and purification. Lyophilized residues of pooled medium or cells were extracted individually with acid–ethanol and the extracts were fractionated by gel filtration employing Sephadex G-75 according to procedures described previously (3). The fraction coinciding with pure bovine insulin (Lilly, Lot PJ4609) and in which the major portion of IRI extractable from hamster pancreatic cell cultures is eluted (3) was purified further by double antibody precipitation (12) employing an aliquot of this fraction not exceeding 1000 μU IRI activity diluted in borate buffer containing 5% bovine serum albumin (BSA), guinea pig anti-porcine insulin serum (Sylvana, Lot 061665-4; Arnel Products, Brooklyn, NY), and rabbit anti- (guinea pig γ-globulin) serum (Arnel Products, Brooklyn, NY). Optimal concentrations of anti-insulin serum and of precipitating antibody were determined experimentally. Insulin regenerated from the double antibody precipitation complex with acid–ethanol was analyzed directly for radioactivity using Bray's solution (13) and a Packard Tricarb liquid scintillation spectrometer, Model 3320 (counting efficiency for ^{3}H approx 20%) or was neutralized with NH_4OH, desiccated *in vacuo,* and redissolved in BSA prior to quantification by radioimmunoassay.

Losses sustained in extraction and purification were determined quantitatively employing a Nuclear Chicago gamma scintillation spectrometer system (Model 1085) and labeled insulin in concentrations approximating those encountered in the present experiments. Recovery of radioactivity averaged 69.7 $\pm$ 3.5% after extraction and Sephadex gel filtration and 43 $\pm$ 2.8% overall for 9 determinations wherein 25, 50, or 100 μU porcine insulin-^{125}I (Abbot, 50–70 mCi/mg) were added to 40 ml aliquots of culture medium and carried through the procedures of extraction and purification described above. Individual values, representing 3 determinations for each insulin concentration employed, were not significantly different from each other within or between concentrations ($p > 0.05$) by two-way analysis of variance without replication (14).

Immunoassay. IRI was measured as previously described (3) using bovine insulin (Lilly, Lot PJ4609) as a standard and suitable aliquots of unfractionated medium from individual culture tubes or purified extracts of pooled medium or cells. The data were corrected for the IRI activity measured in equivalent volumes of identically treated medium in which cells had not been cultured but were not corrected for losses sustained in extraction and purification.

Results. Relationship between AF^{+} granulation and IRI. At 5 days, cultures derived from neonatal hamster pancreas consisted typically of varying admixtures of epithelioid and fibroblastoid cells (Fig. 1A). The epithelioid cells were consistently more numerous and lacked or contained sparse, moderate, or abundant AF^{+} cytoplasmic granules (Fig. 1B). Cultures were well established at this time, and mitotic figures (Fig. 1B) were observed to the maximal extent of 1.3% of the total cell population.

In 45 coverslip preparations representing 5 expts, a statistically significant positive correlation ($r = 0.316$, $0.01 < p < 0.05$) between the rate of IRI secretion and the estimated total number of each AF^{+}-granulated epithelioid cell type was evident on Day 5 only for those cells possessing sparse granulation. The sparsely granulated cells averaged 10.5% of the total AF^{+}-granulated epithelioid cell population compared to 15.7% and 73.8%, respectively, for those possessing abundant or moderate AF^{+} granulation.

IRI biosynthesis. Hamster pancreatic cells cultured in monolayer from Day 5 in the presence of L-leucine-4,5-^{3}H and glucose at 0.8 mg/ml incorporated the label into a pro-

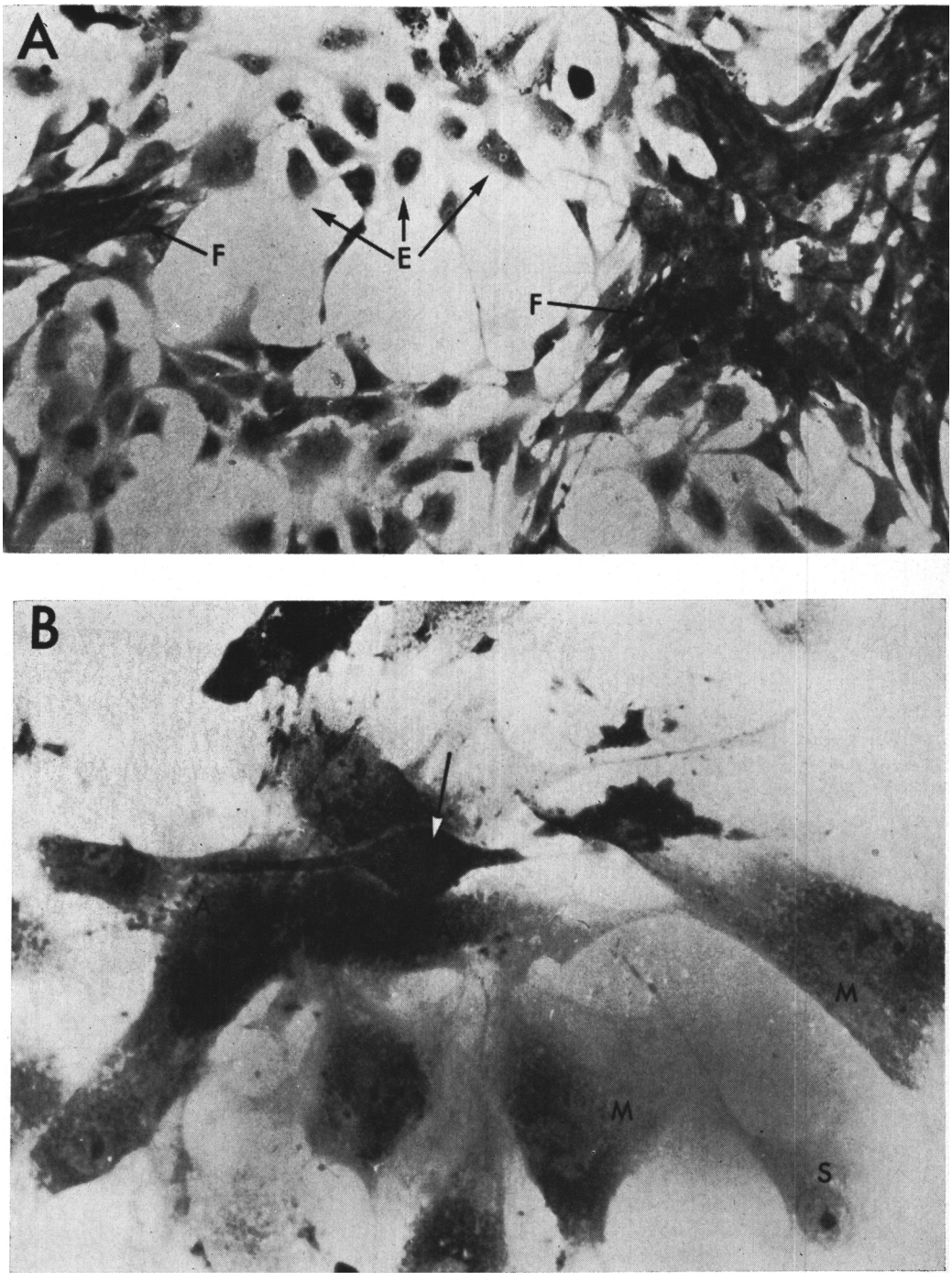

FIG. 1. Primary monolayer cultures derived from neonatal golden hamster pancreas. (A) Five day culture showing admixture of fibroblastoid (F) and epithelioid (E) cells (aldehyde fuchsin, from ×100). (B) Epithelioid cells from a 5-day culture showing abundant (A), moderate (M), and sparse (S) cytoplasmic AF^+ granulation and a mitotic figure (arrow) (aldehyde fuchsin, from ×400).

TABLE I. IRI and cpm in Purified Extracts of Medium and Cells of Hamster Pancreas Cultured in Monolayer in the Presence of L-Leucine-4,5-^{3}H.

Expt. no.	Coverslip preparations pooled for extraction[a] (no.)	Culture medium			Cells			Cell sp act/ medium sp act
		IRI (μU)	cpm	Sp act (cpm/ μU IRI)	IRI (μU)	cpm	Sp act (cpm/ μU IRI)	
1	17	308	1003	3.26	131	855	6.53	2.00
2	19	341	1036	3.04	300	1616	5.39	1.77
3	20	302	1212	4.00	210	2264	10.78	2.70

[a] The coverslip preparations were derived in each experiment from a single tissue pool.

tein possessing a mobility on Sephadex G-75 and an immunological reactivity identical with pure bovine insulin. The IRI activity in the purified extract of the culture medium was greater than that of the corresponding cells in each of three experiments although the specific activity of the IRI from cells was 2–3-fold greater than that from the medium (Table I).

Stimulation of IRI secretion. Table II demonstrates that the average rate of IRI secretion by hamster pancreatic cells cultured in monolayer for 48 hr from Day 5 in the presence of glucose at 3.1 mg/ml or tolbutamide at 100 or 1000 μg/ml was substantially greater than that for glucose at 0.8 mg/ml but not essentially different from the latter in the presence of tolbutamide at 10 μg/ml.

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

Insulinotropic agent	Concn	IRI (μU/ ml/48 hr)
Glucose	0.8 mg/ml	11 ± 1[a] (10)
	3.1 mg/ml	108 ± 32 (16)
Tolbutamide	10 μg/ml	13 ± 2 (14)
	100 μg/ml	30 ± 3 (12)
	1000 μg/ml	32 ± 3 (11)

[a] Mean ± SE for the number of coverslip preparations indicated in parentheses. Coverslip preparations from each of three cultures were used for each different concentration of both insulinotropic agents.

Additionally, the IRI activity in the purified extracts of the medium and corresponding cells of identical cultures was greater consistently in the presence of glucose at 3.1 than at 0.8 mg/ml (Table III). The latter findings suggest that the elevated glucose concentration stimulates synthesis as well as release of IRI by these cultures.

Discussion. The present study provides evidence which indicates that IRI secretion by hamster pancreatic monolayer cultures is most closely associated with a population of epithelioid cells containing relatively few AF$^+$ cytoplasmic granules. This suggests that these are beta cells depleted of stored insulin. As previously shown, AF$^+$-granulated cells in hamster pancreatic monolayer cultures are cytochemically identifiable with pancreatic beta cells (3). They also resemble AF$^+$-granulated cells in islet tissue of the neonatal hamster (15). Unpublished cytochemical and ultrastructural evidence obtained in this laboratory demonstrates additionally that degranulation of AF$^+$-granulated epithelioid cells in identical pancreatic cultures stimulated maximally by glucose or tolbutamide is accompanied by an increase in IRI release. Moreover, AF$^+$ beta cell granules are considered to represent stored insulin or insulin precursor (16).

Incorporation of tritiated leucine into a protein possessing chromatographic and immunological properties identical with those of bovine insulin and release of the labeled protein into the culture medium emphasizes the presence of insulinogenically competent beta cells in these pancreatic cultures at 5–7 days of cultivation and provides reasonable assurance that the IRI released during

TABLE III. Effect of Glucose Concentration on IRI Activity in Purified Extracts of Medium and Cells from Hamster Pancreatic Monolayer Cultures.

Expt. no.	Coverslip preparations pooled for extraction[a] (no.)	Glucose (mg/ml)	IRI (μU) Culture medium	IRI (μU) Cells
1	4	0.8	69	37
		3.1	134	130
2	7	0.8	103	50
		3.1	194	108
3	10	0.8	36	42
		3.1	91	80

[a] The number of coverslip preparations designated for each experiment was used for each glucose concentration. The combined number employed for both glucose concentrations was derived in each experiment from a single tissue pool.

this period of cultivation does not consist entirely of that originally present in the donor islet tissue. Nevertheless, release of substantial quantities of nonlabeled IRI (some of which conceivably may have been formed prior to cultivation) could account for the observed dilution of the specific activity of labeled IRI released by these cultured cells. Additional evidence that these pancreatic monolayer cultures are endocrinologically competent is provided by their responsiveness to glucose and tolbutamide. Moreover, the severalfold increase in cellular IRI content in the presence of elevated glucose concentration strongly suggests stimulation of IRI synthesis. Thus, our data agree with reported findings that monolayer cultures derived from pancreas of other mammalian species are responsive to known insulinotropic agents (6, 9).

Summary. Monolayer cultures derived by enzymatic dispersion of neonatal hamster pancreas consist predominantly of AF+-granulated epithelioid cells, release newly synthesized immunoreactive insulin, and respond to the insulinotropic action of glucose and tolbutamide. These findings provide evidence that endocrinologically competent beta cells are present in these pancreatic monolayer cultures.

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