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Purification of Neonatal Rat Pancreatic Monolayer Cultures for Endocrine Cells (40657)[1]

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Although monolayer cell cultures of the pancreas have been successfully established by several investigators and used in studies of islet hormone release, islet cell morphology, and islet cell replication, fibroblastoid cells which are established in culture along with the endocrine cells rapidly proliferate and eventually overgrow the endocrine cells so as to inhibit their further development. Selective attachment procedures (1), chemically defined medium (2), cystine-free medium (3), addition of sodium ethylmercurithiosalicylate (4, 5), and selective enzymic detachment of endocrine cells from monolayers (6) have all been reported to enrich pancreatic monolayer cultures for endocrine cells. The present studies were undertaken to assess the effectiveness of two additional approaches to this problem.

Medium in which D-valine has been substituted for L-valine has been reported to selectively inhibit fibroblast proliferation while allowing growth of epithelial cells (7). The incorporation into DNA of bromodeoxyuridine added to nutritionally deficient medium is lethal to cells able to proliferate under these conditions, whereas those cells unable to synthesize DNA under such conditions are not destroyed (8). Both of these methods were evaluated for their ability to curtail fibroblast proliferation in pancreatic monolayer cultures.

Materials and Methods. Materials. Trypsin (1:250) was from Difco; type IV collagenase from Worthington; plastic tissue culture dishes from Falcon or Lux; NCTC-135, Medium 199, nonessential amino acid mixture, individual amino acid concentrates, and fetal bovine serum from GIBCO; gentamicin and Eagle's MEM from Microbiological Associates; bromodeoxyuridine (BUdR) from Calbiochem; and D-valine from Sigma.

Preparation of cultures. Pancreases were removed from 3- to 5-day-old Wistar rats, washed at room temperature in Ca- and Mg-free phosphate-buffered saline (PBS) for 5 min, and dissociated by sequential 10-min incubations at 37° in Ca- and Mg-free PBS containing 0.5% trypsin and 0.02% collagenase (1). Both wash and dissociation were aided by stirring with a magnetic stirrer. For each experiment, one pool of neonatal rat pancreases was enzymatically dispersed in this manner. Dissociated cells were collected, washed with medium, and dispensed into 100-mm diameter plastic tissue culture dishes so that each dish received cells from approximately four pancreases. The culture medium usually employed in our laboratory for establishment of these cultures has consisted of (v/v): 45% NCTC-135, 45% Medium 199, and 10% heat-inactivated fetal bovine serum (9–13). This medium is supplemented with glucose (16.5 m*M*) and gentamicin (50 μg/ml). However, in the studies reported here and shown in Tables I and III, culture medium consisted of Eagle's MEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum, nonessential amino acids (14), Earle's salts (15), gentamicin (50 μg/ml), and glucose (16.5 m*M*). Following incubation for 14–16 hr at 37° in a humidified atmosphere of 95% air and 5% CO_2, the cells which were unattached to the culture dishes were collected, the dishes gently rinsed with medium, and the rinses added to the collected cells. Following centrifugation, equal aliquots of cells were dispensed into 60-mm diameter plastic tissue culture dishes so that each dish contained cells from approximately one pancreas. However, results from one experiment may not be similar to those from another experiment since different pools of neonatal rat pancreas

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TABLE I. DNA CONTENT, INSULIN SECRETION, AND GLUCAGON SECRETION BY TREATED AND CONTROL CULTURES

Day of culture	Day after start of culture treatment[a]	DNA (μg/culture ± SE) (n)			Insulin (μU/ml ± SE) (n)			Glucagon (pg/ml ± SE) (n)		
		Control	D-Val	BUdR	Control	D-Val	BUdR	Control	D-Val	BUdR
6	4	5.08 ± 0.11 (3)	0.41 ± 0.02 (4) ***[b]	0.56 ± 0.05 (4) ***	1280 ± 24 (3)	1265 ± 17 (4) NS[b]	1200 ± 140 (4) NS	1673 ± 59 (3)	975 ± 27 (4) ***	1240 ± 37 (4) ***
9	7	11.28 ± 0.36 (4)	0.34 ± 0.14 (3) ***	2.16 ± 0.03 (4) ***	1698 ± 63 (4)	1083 ± 17 (3) ***	1340 ± 37 (4) ***	1300 ± 38 (4)	693 ± 138 (3) ***	1365 ± 38 (4) NS

[a] BUdR (10^{-5} *M*) and D-valine (92 mg/liter) were started on culture Day 2 (culture treatment Day 0). D-Valine was continued throughout the treatment period, but BUdR was added only for the first 24 hr, following which control medium was added. Control medium was Eagle's MEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum, nonessential amino acids, gentamicin (50 μg/ml), and glucose (16.5 m*M*). D-Val medium was similar to control medium except D-valine was substituted for L-valine, and fetal bovine serum was dialyzed. BUdR medium was also similar to control except BUdR (10^{-5} *M*) was added for the first 24 hr (until culture treatment Day 1).

[b] When compared to control, $P < 0.001$ (***), P = not significant (NS).

were used and the number of cells dispersed to each culture dish could differ between experiments. Tables I, II, and III give further experimental details for separate experiments.

Assays. Immunoreactive insulin (16), immunoreactive glucagon (17), and DNA (18) were measured using methods and materials described previously. Data in Table I were examined by analysis of variance using orthogonal contrasts (19), and by Newman–Keul's multiple comparisons procedure (20). Data in Tables II and III were examined by one-way analysis of variance and the Newman–Keul's procedure.

Results. When pancreatic monolayer cultures were maintained in control medium for 9 days, both insulin and glucagon secretion remained quite constant whereas the DNA content of the cultures increased substantially (Table I). It was apparent from examination of cultures in a phase-contrast microscope that this increase in DNA content was due almost entirely to fibroblast proliferation.

When either D-valine-substituted medium or BUdR treatment were used, cell proliferation was markedly curtailed as indicated in Table I by the significant reduction in DNA content on culture Days 6 (8% and 11% of control) and 9 (3% and 19% of control). On the other hand, both insulin and glucagon release were not affected to a comparable degree. For example, on culture Day 6, DNA was reduced to 8 and 11% of control with D-valine medium and with BUdR treatment, respectively, whereas glucagon release was reduced to only 58 and 74% of control for D-valine and BUdR, respectively, and insulin release was virtually unchanged from control. Furthermore, when cultures were maintained in D-valine medium, culture growth remained curtailed as DNA content remained constant, but both insulin ($P < 0.01$) and glucagon ($P < 0.001$) release were reduced further. On the other hand, following BUdR treatment for 24 hr cultures recovered their proliferative capacity as DNA increased fourfold between culture Days 6 and 9 ($P < 0.001$), and insulin ($P < 0.05$) and glucagon ($P < 0.025$) release increased slightly.

B-Cell function was examined further in D-valine medium and following BUdR treatment. Glucose-stimulated insulin release was maintained on culture Day 6 following two

TABLE II. INSULIN SECRETION BY BUdR-TREATED CULTURES[a]

Hour[b]	Glucose (m*M*)	*n*	Insulin (μU/ml ± SE)	*P*[c]
1	1.7	6	413 ± 29	
2	1.7	6	487 ± 44	
3	1.7	6	448 ± 11	
4	1.7	6	415 ± 24	
5	16.5	6	764 ± 52	<0.001

[a] Cultures were incubated in Eagle's MEM containing glucose (5.5 m*M*), gentamicin (50 μg/ml), 10% (v/v) heat-inactivated fetal bovine serum, and BUdR (10^{-5} *M*) for 24 hr on culture Day 2 and again on culture Day 4. Following each incubation with BUdR, cultures were exposed to fluorescent light for 90 min. Cultures were incubated in 45% (v/v) NCTC-135, 45% (v/v) Med 199, and 10% (v/v) heat-inactivated fetal bovine serum containing glucose (16.5 m*M*) and gentamicin (50 μg/ml), between incubations in BUdR medium.

[b] On culture Day 6, cultures were incubated for successive 1-hr periods in 45% (v/v) NCTC-135, 45% (v/v) Med 199, and 10% (v/v) heat-inactivated fetal bovine serum containing glucose at the indicated concentrations, and gentamicin (50 μg/ml), and insulin released into the medium during each 1-hr period was measured. Medium was changed hourly.

[c] *P* vs all other groups.

TABLE III. INSULIN SECRETION IN D-Val MEDIUM[a]

Hour[b]	Glucose (mM)	Experiment 1 n	Experiment 1 Insulin (μU/ml ± SE)	Experiment 1 P[c]	Experiment 2 n	Experiment 2 Insulin (μU/ml ± SE)	Experiment 2 P[c]
1	1.7	2	733 ± 13		10	127 ± 4	
2	1.7	2	613 ± 13		10	150 ± 4	
3	16.5	2	1378 ± 12	<0.001	10	333 ± 15	<0.001
4	1.7	2	568 ± 47		10	133 ± 10	

[a] Cultures were incubated in Eagle's MEM containing glucose (16.5 mM), gentamicin (50 μg/ml), nonessential amino acids, 10% (v/v) heat-inactivated and dialyzed fetal bovine serum, and D-Val (92 mg/liter) substituted for L-Val.

[b] On culture Day 8, cultures were incubated for successive 1-hr periods in Eagle's MEM containing glucose at the indicated concentrations and other additions as noted above, and insulin released into the medium during each 1-hr period was measured. Medium was changed hourly.

[c] P vs all other groups.

24-hr treatments with BUdR (Table II). Likewise, glucose-stimulated insulin release was evident on culture Day 8 in cultures that had been maintained in D-valine medium (Table III).

Discussion. The usefulness of pancreatic monolayer cell cultures in the long-term study of islet cell function has been limited due to the rapid proliferation of fibroblastoid cells. The rapid growth of fibroblasts is undesirable not only due to their mechanical crowding effect upon the endocrine cells but also because of their consumption of nutrients. Although a number of methods have been reported to be useful in decreasing the number of fibroblasts in these cultures, the mechanisms by which these effects were produced remain unexplained.

It has been clearly demonstrated in the studies reported here that cell proliferation was reduced much more than either insulin or glucagon release under the culture conditions tested. It has been previously reported that only cells containing D-amino acid oxidase can convert D-valine to the essential L-enantiomer (7). We have not been able to histochemically confirm the presence of D-amino acid oxidase in these cultured islet cells (unpublished). Nevertheless, it is apparent that whereas culture DNA content is greatly reduced by substitution of D-valine for L-valine, both glucagon and insulin release are not reduced as much. This suggests that A- and B-cell function have not been affected as much as culture growth by maintenance of pancreatic cells in this medium. These findings as well as the previously reported effects of chemically defined medium (2), cystine-deficient medium (3), and addition of sodium ethylmercurithiosalicylate (4, 5) suggest that a common feature underlying these purification approaches may be the apparently less fastidious requirement for certain medium constituents by the endocrine cells than the more rapidly proliferating fibroblasts. This may also explain why these inhibitory conditions have been observed to be less effective when the fibroblasts become confluent and contact inhibited, a situtation in which fibroblast proliferation is reduced.

The successful application of BUdR is also based upon the property of the fibroblasts to proliferate more rapidly than do the endocrine cells. Following incubation of subconfluent pancreatic monolayer cultures for 24 hr with [^{3}H]thymidine, greater than 95% of the fibroblasts exhibit labeling of nuclei compared to a 5–7% labeling index for endocrine cells (unpublished (21, 22)). Like thymidine, BUdR is incorporated into DNA by proliferating cells, and this proves lethal when these cells are subsequently exposed to fluorescent light (8). Thus when subconfluent pancreatic cultures are treated for 24 hr with BUdR, preferential incorporation of BUdR by fibroblasts may be expected to lead to a loss of greater than 95% of the fibroblasts, compared to a loss of about 5% of the islet cells. Furthermore, when BUdR-treated cultures are subsequently maintained in control medium, recovery of proliferation may be expected, as indicated by a rise in DNA content, while islet cell numbers are expected to remain quite constant, as indicated by amounts of insulin and glucagon released. Indeed this appeared to be the case when the

data in Table I was examined. Although not tested specifically, these results suggest that sequential treatments with BUdR may be more useful than a single treatment for the continued reduction of fibroblast proliferation in these pancreas cultures.

Summary. The use of either D-valine-substituted medium or BUdR treatment have been shown to be effective in reducing fibroblastoid cell proliferation in pancreatic monolayer cell cultures established from neonatal rats. Maintenance of these cultures in D-valine medium appeared to completely curtail further culture growth, but this was apparently accompanied by a loss of both A and B cells as evidenced by a fall in both glucagon and insulin release. A single 24-hr treatment of cultures with BUdR was just as effective in curtailing fibroblastoid cell growth, but within a few days following such treatment, fibroblast proliferation resumed, although the number of fibroblasts remained reduced compared to control cultures. On the other hand, with BUdR treatment continued loss of A and B cells apparently did not occur.

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