REMOVAL OF FIBROBLASTOID CELLS FROM PRIMARY MONOLAYER CULTURES OF RAT NEONATAL ENDOCRINE PANCREAS BY SODIUM ETHYLMERCURITHIOSALICYLATE*

Jan T. Braaten, Michael J. Lee, Antoinette Schenk and Daniel H. Mintz

Division of Endocrinology and Metabolism, Department of Medicine, University of Miami School of Medicine Miami, Florida 33152

Received September 30,1974

SUMMARY. Experimental use of primary cultures of endocrine pancreas is constrained by early, vigorous proliferation of fibroblastoid cells. This report demonstrates that the addition of sodium ethylmercurithiosalicylate (thimerosal) to the culture media can selectively destroy these fibroblastoid cells. The cytotoxicity of this mercurial compound was also demonstrated with other fibroblast-like cell lines. Treatment of primary cultures of the endocrine pancreas with sodium ethylmercurithiosalicylate yielded highly enriched, morphologically intact, functionally competent endocrine cells that were capable of cell replication.

Monolayer cell cultures of endocrine pancreas (1-5), although possessing secretory patterns for insulin (1-5), glucagon (4, 5) and gastrin (5), have only limited use beyond seven to 10 days because of the rapid proliferation of fibroblastoid cells which envelop the monolayer clusters of endocrine cells, inhibiting their further expansion on the culture dish. The present report details methods for selective removal of the fibroblastoid cells from these primary cultures of neonatal rat pancreas and the potential application of these methods to fibroblastoid cells found in other types of monolayer cell cultures.

METHODS AND MATERIALS. Cultures were established by two procedures using pancreases obtained from three to four-day-old Wistar-Lewis rats (Charles River). In one, the pancreas was enzymatically dissociated with a trypsin

*Supported in part by National Institutes of Health grant No. AM-14106 and the Florida Juvenile Diabetes Research Foundation.

collagenase mixture as described by Lambert et al. (3). In the other, whole islets were isolated (6) and further purified on Ficoll gradients (7). The dispersed cells or whole islet pellets were suspended in culture media and seeded in 60 mm plastic culture dishes (Falcon) at a density of cells or whole islets representing a yield of 2.5 or 5-7 pancreases per dish, respectively. The culture medium was 199 with unmodified Earle's salts, 10% fetal calf serum (both from Grand Island Biological Company, Calif.), glucose 3 mg/ml, aqueous sodium penicillin 400 U/ml and amphotericin B 1.5 mcg/ml and replaced every 48 hours.

Radioimmunoassay was used to measure insulin (8) and glucagon (9).

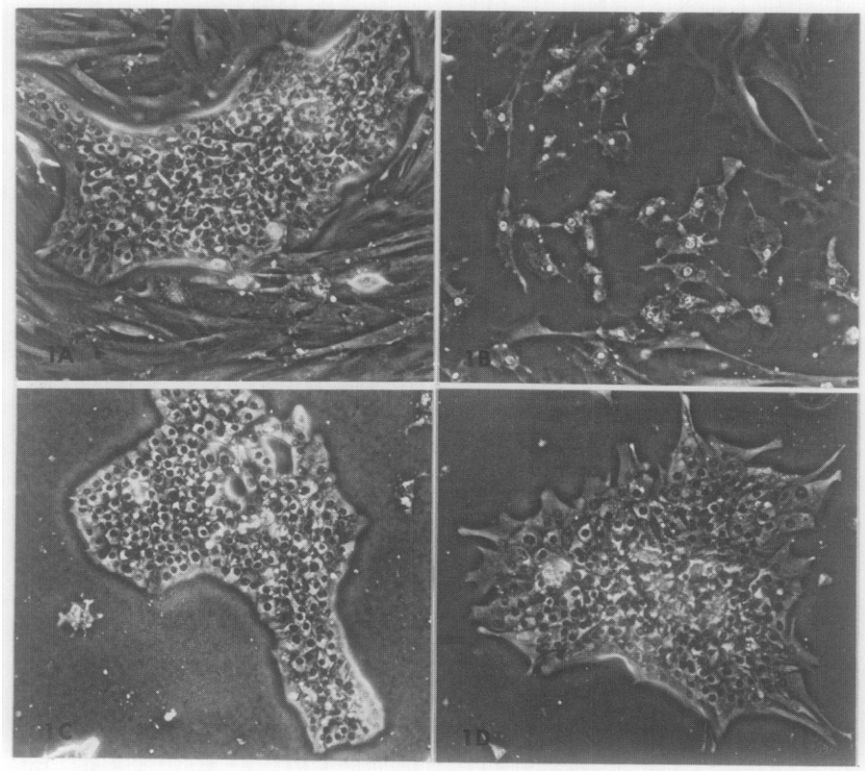

Fig. 1. Monolayer cultures of rat neonatal pancreas, seeded from cells after dissociation with trypsin-collagenase. Magnification X350. A: Cluster of endocrine cells surrounded by fibroblastoid cells after five days in culture. B: Nuclear and cytoplasmic degeneration of fibroblastoid cells 24 hours after application of thimerosal, 1.15 µg/ml. C: All fibroblastoid cells have disappeared, leaving behind a cluster of endocrine cells seven days after application of thimerosal, 1.15 µg/ml. D: Cluster of endocrine cells five days after removal of thimerosal. Protoplasmic projections are prominent at the border of the cluster.

Table 1. INSULIN AND GLUCAGON RELEASE IN CONTROL AND THIMEROSAL-TREATED MONOLAYER CULTURES OF NEONATAL PANCREATIC ISLETS*

	Day of Culture		5	7	10	12	14	17	19
INSULIN mU/dish/2 hrs (mean ± SEM)	Control	n=6	12.1± 2.5	23.5±2.9	29.3±5.0	28.2±2.2	27.9±1.9	29.2±1.3	17.6±1.3
	Thimerosal-treated†	n=10	10.6±1.8	24.3±1.7	40.6±3.4	56.9±8.0	80.4±6.5	54.3±8.3	67.8±10.5
	p value		ns	ns	ns	<.02	<.001	<.05	<.005
GLUCAGON ng/dish/2 hrs (mean ± SEM)	Control	n=6	1.50±.12	2.16±.31	2.26±.50	2.09±.38	1.83±.19	1.46±.12	1.32±.06
	Thimerosal-treated†	n=10	2.57±.22	3.77±.71	4.37±.53	8.66±1.98	7.13±1.53	5.46±1.45	5.21±1.16
	p value		<.005	ns	<.02	<.02	<.02	<.05	<.025

*The cultures were established from neonatal pancreatic islets isolated with collagenase and purified on a discontinuous Ficoll gradient. The hormone levels represent amounts released during a 2-hour incubation, performed three times a week, in a complete culture medium.

†Thimerosal, 1.15 µg/ml, was removed from the culture media during the two-hour incubation studies.

Cell morphology was assessed by phase contrast microscopy, light microscopy of beta cells stained with an aldehyde thionine procedure (10), as modified by Dr. B. Blondel (personal communication), and electron microscopy utilizing techniques for tissue preparation to be reported elsewhere.

RESULTS. Natural development of pancreatic cultures. After seeding dissociated cells, numerous endocrine cell clusters, containing predominantly well-granulated beta and a smaller number of alpha and delta cells, were identified within two to three days. By three to five days, proliferating fibroblastoid cells formed a confluent layer which enveloped the scattered monolayered clusters of endocrine cells (Fig. 1A). Fibroblastoid cells continued to proliferate, inhibiting any further spreading of the endocrine cell clusters. After 10 to 14 days, these clusters retracted into ovoid or round multilayered bodies, the centers of which frequently contained necrotic cells. In spite of these changes in topography, continued release of insulin and glucagon could still be demonstrated (Table 1). Similar observations were made with the isolated whole islet separation procedure, although the fibroblastoid cells grew to confluence at a slower rate.

Selective removal of fibroblastoid cells. The addition of 0.46 to 2.3 µg/ml of sodium ethylmercurithiosalicylate (thimerosal)(Aldrich Chemical Company, Inc.) to culture media, two to four days after initial seeding for the dissociated cell preparations and 24 hours earlier for the whole islet preparation, selectively detached the fibroblastoid cells from the culture plate. The concentration of thimerosal and the timing of application were important variables. Earlier application or higher concentrations adversely effected the development of the endocrine cells whereas application after the fibrobalstoid cells had grown to density, had little effect on their detachment from the culture dish.

The earliest effect was evident 18 to 24 hours after application of thimerosal. The fibroblastoid cells underwent nuclear and cytoplasmic

degeneration, preceding their detachment from the dish (Fig. 1B). After seven days of continuous exposure to thimerosal, large areas of the dish were free of fibroblastoid cells leaving behind clusters of endocrine cells firmly attached (Fig. 1C). Continuous exposure to thimerosal for 18 days was associated with detachment of some endocrine cells, but those remaining were ultrastructurally intact and continued to release significantly greater amounts of insulin and glucagon into the media compared with untreated controls (Table 1) (Fig. 3).

When thimerosal was withdrawn from the cultures after continuous exposure for 7 to 18 days, the monolayer endocrine cell clusters gradually expanded, and cytoplasmic projections (Fig. 1D) from beta cells, identified by aldehydethionine stain, eventually bridged with adjacent endocrine cell clusters (Fig. 2), suggesting proliferation of these cells. This was further supported by the finding that the addition of colcemid (0.03 μg/ml for 24 hours) to 18-day-old cultures three days after removal of thimerosal, revealed numerous aldehyde-thionine positive cells in metaphase.

Exposure of stock cell lines of fibroblast-like cultures derived from human neonatal skin (International Scientific Industries, Inc.), normal human skin (CRL 1119), human embryonic lung (WI-38), baby hamster kidney (BHK-21; C13) and mouse connective tissue (NCTC, C-929) (American Type Culture Collection) to increasing amounts of thimerosal (0.46 to 1.7 μg/ml) produced a dose-dependent progressive necrosis of all cells tested.

DISCUSSION. Addition of thimerosal, to primary cultures of neonatal pancreas, resulted in the selective removal of the fibroblastoid cells from the cultures. This organic mercurial is thought to act through reversible inhibition of sulfhydryl enzymes (11). The mechanism of the toxic effects on the fibroblastoid cells in these cultures remains for future study. Of primary importance, however, was the fact that the endocrine cells maintained normal function as evidenced by the secretion

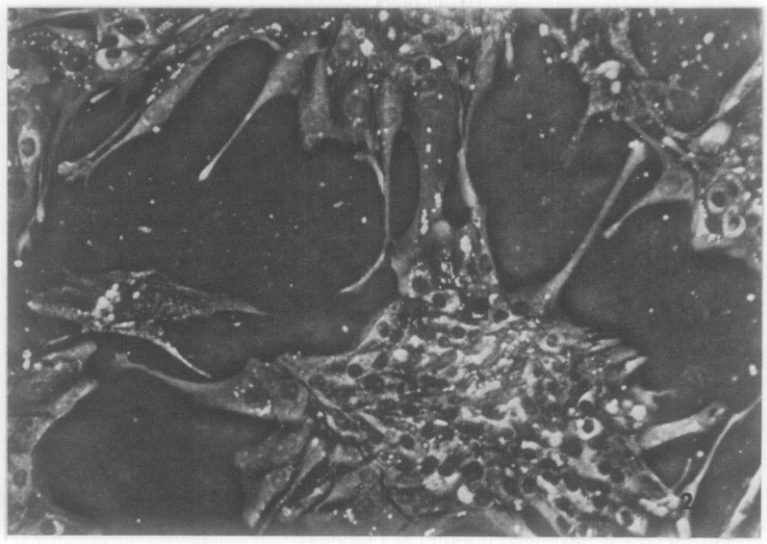

Fig. 2. Endocrine cells from one cluster are sending long protoplasmic projections to adjacent clusters, forming bridges. Magnification X700.

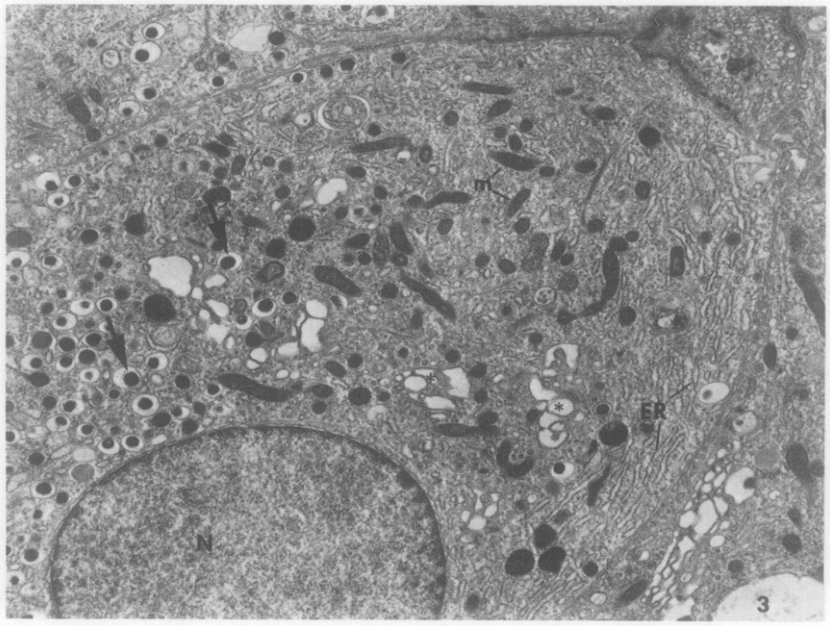

Fig. 3. Electron micrograph showing a portion of a beta cell from an islet exposed to thimerosal, 1.15 µg/ml, for 18 days. Arrows point to beta granules characterized by a central dense core surrounded by a translucent halo. N = nucleus, ER = cisternae of the endoplasmic reticulum, m = mitochondria, asterisk = Golgi complexes. Magnification X10,000.

of insulin and glucagon into the medium. In addition, the morphology of the endocrine cells evaluated by both phase contrast and electron microscopy during continuous exposure of the cultures to thimerosal, was preserved. Withdrawal of the thimerosal was associated with spreading of the monolayer endocrine cell clusters, protoplasmic bridging to adjacent clusters, entry into mitosis of the endocrine cells and maintained functional secretory capability.

The observations reported provide a markedly improved, virtually pure culture of functioning pancreatic endocrine cells for continued study. Moreover, the cytotoxic effect of thimerosal on fibroblastoid cells from several tissues suggests that it may have general applicability to other primary mammalian cultures in which overgrowth of fibroblasts prevent establishment of pure or clonal cell lines.

ACKNOWLEDGEMENTS. We gratefully acknowledge the technical assistance of Mrs. Carol Quigley and Miss Ulla Jarlfors. We also acknowledge the donation of the fibroblastoid-like cell lines from Dr. Sheldon Greer, and antibody 30K from Dr. Roger Unger.

REFERENCES

1. Hilwig, I., Schuster, S., Heptner, W. and Wasielewski, E.v. (1968) Z. Zellforsch. 90, 333-346.
2. Macchi, I.A. and Blaustein, E.H. (1969) Endocrinology 84, 208-216.
3. Lambert, A.E., Blondel, B., Kanazawa, Y., Orci, L. and Renold, A.E. (1972) Endocrinology 90, 239-248.
4. Marliss, E.B., Wollheim, C.B., Blondel, B., Orci, L., Lambert, A.E., Stauffacher, W., Like, A.A., and Renold, A.E. (1973) Europ. J. Clin. Invest. 3, 16-26.
5. Braaten, J.T., Schenk, A., Lee, M.J., McGuigan, J.E. and Mintz, D.H. (1974) J. Clin. Invest. 53, 10a.
6. Lacy, P.E. and Kostianovsky, M. (1967) Diabetes 16, 35-39.
7. Lindall, A., Steffes, M. and Sorensen, R. (1969) Endocrinology 85, 218-223.
8. Herbert, V., Lau, K-S., Gottlieb, C.W. and Bleicher, S.J. (1965) J. Clin. Endocr. 25, 1375-1384.
9. Aguilar-Parada, E., Eisentraut, A.M. and Unger, R.H. (1969) Am. J. Med. Sci. 257, 415-419.
10. Paget, G.E. (1959) Stain Tech. 34, 223-226.
11. Esplin, D.W. (1965) in The Pharmacological Basis of Therapeutics. 3rd Ed. (L.S. Goodman and A. Gilman, eds.) pp. 1038-1039, The Macmillan Company, New York.