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Cell Biology

Continuous, clonal, insulin- and somatostatin-secreting cell lines established from a transplantable rat islet cell tumor

[athymic *nude* mice/APUD (amine-handling) cells/insulinoma]

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ABSTRACT **Continuous cell lines that secrete both insulin and somatostatin were established by two cooperating groups of investigators from a serially transplantable, radiation-induced, rat islet cell tumor. The cell lines, named RIN-r and RIN-m, were initiated from tumors maintained in inbred rats or in athymic *nude* mice, respectively. The cultured cells are epithelioid, free of fibroblast contamination, and can be cloned. They have a hypodiploid chromosome number, are tumorigenic, and possess amine-handling properties, including high levels of L-dopa decarboxylase and formaldehyde-induced fluorescence. Preliminary analysis of clones revealed a spectrum of insulin secretion from undetectable to relatively high. These clonal cell lines provide important systems to study the biology of insulin and somatostatin.**

Continuous cultures of pancreatic islet cells provide a potentially valuable tool for diabetes research. Although functioning islet cell cultures from humans and rodents have been maintained for various periods of time (1–3), they have consisted of mixtures of cell types and have eventually been overgrown by fibroblasts. Likewise, functioning cultures of islet cell tumors have had relatively limited replicative ability and have not been established as continuous clonable cell lines (4, 5). In this report we describe the establishment and cloning of a transplantable rat islet cell tumor. Both the tumor and the cultured cells derived from it secrete insulin and somatostatin.

MATERIALS AND METHODS

Origin of the cultures. Cultures were initiated from a transplantable islet cell tumor (6) induced by high-dose x-irradiation in an inbred NEDH (New England Deaconess Hospital) rat. The tumor was maintained by serial transplantation in NEDH rats. After nine transplants, it was successfully heterotransplanted into athymic *nude* mice, BALB/c background (ARS/Sprague–Dawley, Madison, WI). Continuous cell lines were derived either from rat transplants (Joslin group) or from *nude* mouse heterotransplants (NCI–VA group). These cell lines were named RIN-r and RIN-m, respectively.

Establishment of RIN-r Cell Line. Tumors were removed aseptically from recipient rats and cut into small fragments. The tumor cells were separated from the connective tissue stroma by washing the fragments with tissue culture medium 199 containing 0.1% fetal bovine serum, 16.5 mM glucose, and 400 units of penicillin per ml. The culture medium used throughout the remainder of the procedure was similar, but contained 10% fetal bovine serum. Erythrocytes present in the original cell suspension were removed by centrifugation (750 × *g*, 10 min) on a discontinuous gradient consisting of a solution of 25% dialyzed Ficoll in culture medium overlaid with culture medium alone. The tumor cells were collected from the interface, washed, and plated in 150-mm plastic dishes at a density of approximately 1×10^6 cells per ml.

After a number of unsuccessful attempts to establish continuous cultures, we elected to enhance growth and attachment of tumor cells by plating them on feeder layers of a rat liver cell line, BRL3A (obtained from H. G. Coon) (7) previously exposed to 6000 rads (60 grays) of x-irradiation. Cells were detached by incubation with a solution of 0.05% trypsin/0.02% EDTA in calcium- and magnesium-free phosphate-buffered saline after 2–3 weeks and transferred to fresh feeder layers. One of these experiments eventually led to the development of cell line RIN-r. In this instance, tumor cells were maintained on feeder layers for 3 months, after which they continued to attach to the substrate and replicate slowly without use of feeder layers. At this stage, the epithelioid tumor cells were contaminated with large numbers of fibroblastoid cells. Approximately 4×10^7 cells were injected subcutaneously into the interscapular area of an NEDH rat. The tumor that formed was recultured without the aid of feeder layers. The resultant monolayers still contained many fibroblastoid cells, which were eliminated by a combination of three maneuvers. First, exposure of the cultures to trypsin/EDTA solution for 1 min tended to selectively dislodge the tumor cells, which were subsequently transferred to new dishes. Second, the fibroblastoid cells reattached faster than epithelioid cells, and the unattached cells were transferred to fresh dishes after a few hours. Third, cultures were exposed to cystine-free Eagle's minimal essential medium for 48 hr, which induced selective fibroblastoid cell necrosis (8). These techniques resulted in the establishment of a continuous epithelioid cell line apparently free of fibroblastoid cell contamination. The cells were subcultured at a ratio of 1:2 every 7 days.

Establishment of RIN-m Cell Line. The RIN-m cell line was established from the fourth *nude* mouse heterotransplant. Cultures from earlier transplants resulted only in temporary growth of epithelioid cells. Cells were prepared as described above and seeded into 75-cm^2 flasks in either Dulbecco's modification of Eagle's minimal essential medium (DMEM) or RPMI-1640 medium supplemented with 10% fetal bovine serum. Epithelioid and fibroblastoid cells were present in all flasks. After 9 days the cells were exposed to cystine-free medium for 16 hr. Fibroblastoid cells of murine origin were completely eliminated and appeared more susceptible to this technique than the fibroblastoid cells of rat origin present in early RIN-r cultures. Progressive growth of epithelioid cells occurred in both media, but more rapidly in DMEM. Initially, RIN-m cells were subcultured at a 1:2 ratio. After the fourth

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Abbreviation: DMEM, Dulbecco's modification of Eagle's minimal essential medium.

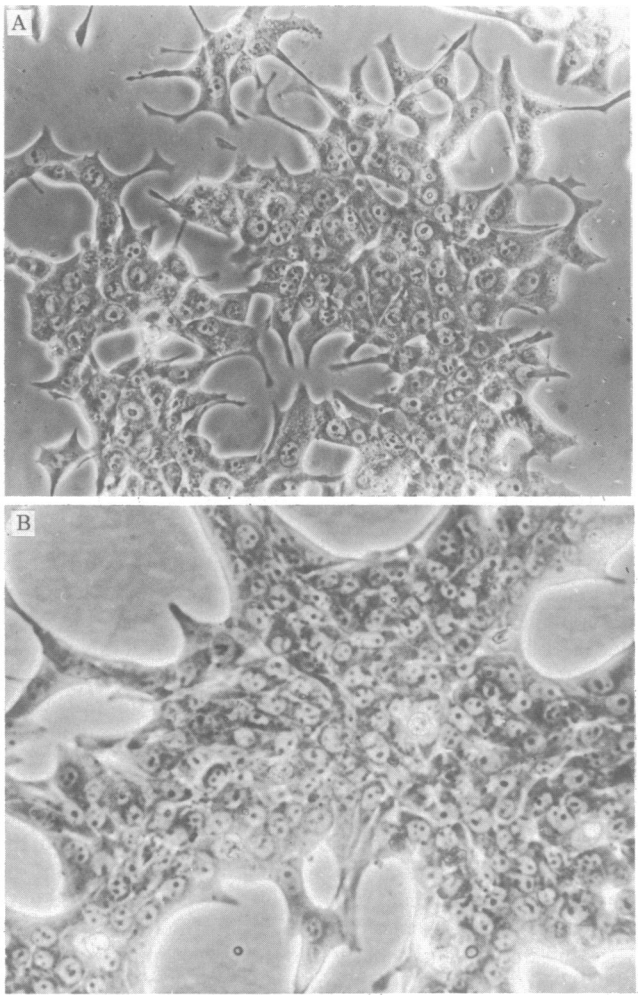

FIG. 1. Phase-contrast photomicrographs of RIN cells. The cells are epithelioid, and cytoplasmic processes extend from free cell surfaces. (*A*) RIN-r cells. (×120); (*B*) RIN-m cells (×210).

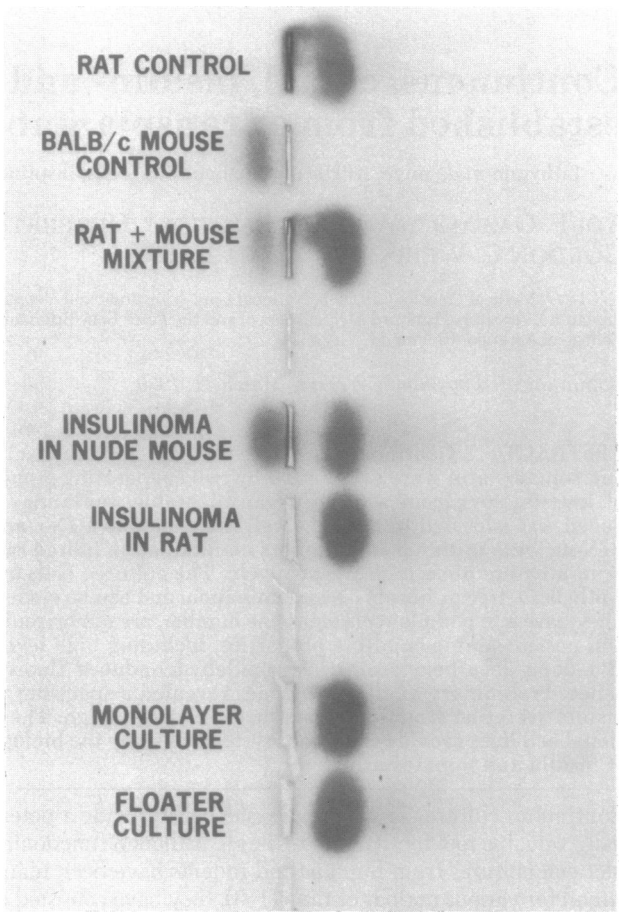

FIG. 2. Starch gel electrophoresis of glucosephosphate isomerase isoenzymes. Rat tumors and floating and adherent RIN-m cultures consist of the rat form of the isoenzyme. *nude* mouse tumors, consisting of rat tumor cells supported by mouse stromal cells, contain both rat and mouse forms. The NEDH rat form migrates towards the anode (to the right of the origin), while the BALB/c *nude* mouse form migrates towards the cathode (to the left of the origin).

passage, 5×10^6 cells were seeded into 75-cm^2 flasks; they reached densities of $1.5–2.0 \times 10^7$ cells after seven days.

Characterization of Cultured Cells. The parent cultures were cloned by seeding 100 or 1000 cells into 60-mm dishes. After 14–21 days, well-isolated colonies were harvested with the use of cloning cylinders and propagated to mass culture. Tumorigenicity was tested by injecting 1×10^5 to 1×10^7 cells into NEDH rats (RIN-r cells) or athymic *nude* mice (RIN-m cells) by the subcutaneous or intraperitoneal routes. For chromosome counts, actively growing cells were treated with colchicine (0.02 μg/ml), centrifuged, resuspended in 0.075 M potassium chloride for 10 min, fixed in absolute alcohol/acetic acid (3:1, vol/vol), spread on alcohol-treated slides, and stained with Giemsa. Thirty well spread metaphases were counted.

Cell pellets of cultures and tumor homogenates were tested for mouse and rat forms of the enzymes glucose-phosphate isomerase (EC 5.3.1.9) and malic enzyme, soluble form (EC 1.1.1.40) by starch electrophoresis as described (9). Cell pellets of actively growing cells were assayed for L-dopa decarboxylase (aromatic-L-amino-acid decarboxylase, EC 4.1.1.28) activity by a modification (10) of a previously published method (11). Activity is expressed as nmol of CO_2 released from L-[^{14}C]-dopa/hr per mg of protein. For formaldehyde-induced fluorescence (12), cell suspensions were placed on glass slides, air dried in a desiccator for 1 hr, and incubated at 80°C for 3 hr in a sealed container with paraformaldehyde powder, relative humidity 70%. Slides were examined in a Leitz Orthoplan microscope with epi-illumination (excitation wavelength 355–425 nm, barrier filter 460 nm). Tests for mycoplasma contamination were performed by Microbiological Associates (Bethesda, MD). Infection with murine leukemia virus was tested by assays for RNA-dependent DNA polymerase and fluorescent antibody assays for viral group-specific antigen as described (13).

Hormone levels were measured by radioimmunoassay. Insulin levels were determined by a double antibody method using purified rat insulin standards (14). Somatostatin levels were determined by a modification (15) of the method of Patel and Reichlin (16). The antiserum used, D6, gave values identical to those with the Patel antiserum R149 (17). Glucagon was measured by using antiserum 30K (purchased from Roger Unger) (18).

RESULTS

Characteristics of Cultured Cells. RIN-r cultures consisted of epithelioid cells without apparent fibroblastoid contamination (Fig. 1*A*); their appearance was similar to that of RIN-m cells (Fig. 1*B*). The population doubling time was approximately 52 hr in medium 199 with 10% fetal bovine serum. The cells had a hypodiploid chromosome number ($2n = 42$) with a modal number of 40 (39–41) when analyzed at passage 43. The parent culture could be cloned at low efficiency (<1%) in this medium. Approximately 6×10^7 cells injected subcuta-

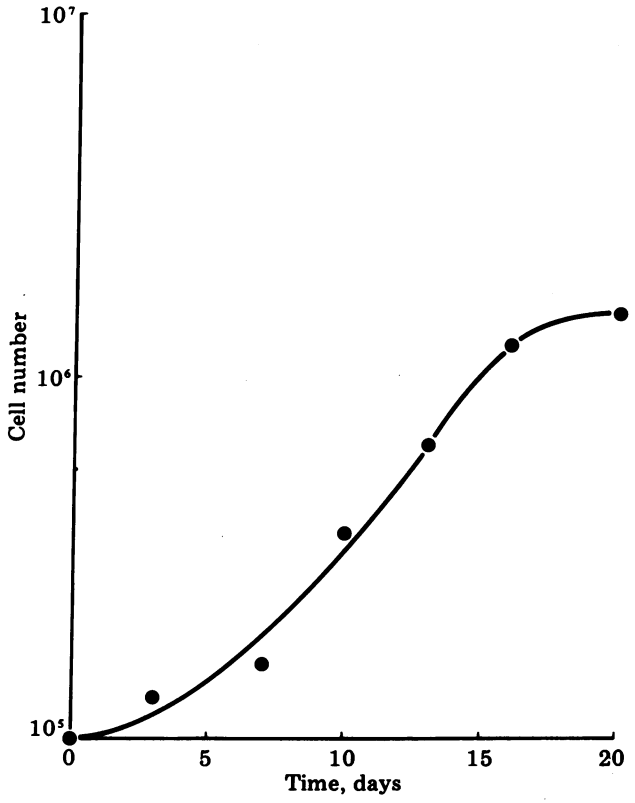

FIG. 3. Growth curve of RIN-m cells in DMEM. The population doubling time was 75 hr.

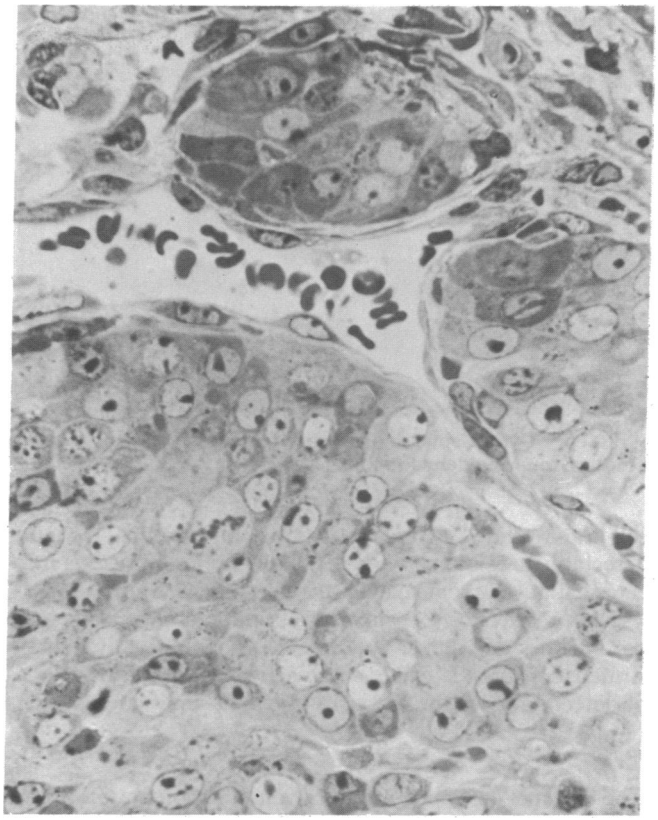

FIG. 4. Microscopic appearance of subcutaneous tumor induced in an athymic *nude* mouse after injection of RIN-m cells. The tumor cells grow in cords and bundles, in a highly vascular loose connective tissue stroma. (Epon embedded, toluidine blue stain, 1-μm section; ×1000.)

neously into six NEDH rats gave rise to tumors weighing several hundred milligrams within 4–5 weeks. Animals became hypoglycemic, with plasma glucose levels ranging from 19 to 41 mg/dl, with a mean of 30 (SEM ±3) mg/dl. Metastases were not grossly visible. The extractable insulin content of these tumors ranged from 0.37 to 4.68 international units (U)/g (wet weight), with a mean of 2.48 (SEM ±0.69) U/g (wet weight). RIN-r cells were free of contamination with mycoplasma.

As noted above, the appearance of RIN-m cells was identical to that of RIN-r cells (Fig. 1*B*). The absence of stromal cells of mouse origin in RIN-m cultures was confirmed by isoenzyme analyses, which demonstrated the presence of rat and the absence of mouse forms of the enzymes, whereas *nude* mouse heterotransplants contained both forms (Fig. 2). Population doubling times were 60–80 hr (Fig. 3) and varied with culture medium and length of culture time. Growth rates were faster in DMEM than in RPMI-1640 medium, and also faster at later than at earlier culture passages. RIN-m cells had a hypodiploid chromosome number ($2n = 42$), with a modal number of 40 (37–42) at both early and late culture passages. The parent sublines could be cloned at low efficiencies (0.1%) in liquid and semisolid media. Harvested clones (from both RIN-r and RIN-m cultures) grew vigorously and could be propagated to mass cultures. RIN-m cells were free of contamination with mycoplasma and murine leukemia virus.

Groups of four or five *nude* mice were injected subcutaneously or intraperitoneally with 1×10^5, 1×10^6, or 1×10^7 RIN-m cells. All of the injected mice developed tumors or ascites 12–48 days later. Unless electively sacrificed, the mice died 3–4 weeks later, presumably of hypoglycemia. The subcutaneous tumors were circumscribed and noninvasive; intraperitoneal tumors were multiple masses attached to the peritoneal surfaces of the abdominal viscera. Histologically (Fig. 4) the tumors closely resembled the transplanted rat tumor from which they were derived originally. Distant metastases were not detected grossly or microscopically.

RIN-m cell pellet had a high L-dopa decarboxylase level (289 nmol of CO_2/hr per mg of protein); control cultures (BRL3A, rat, and mouse fibroblastoid cultures) had activities only 1–0.1% of this. After exposure to formaldehyde vapor, the cells had bright yellow-green fluorescence in their cytoplasms (Fig. 5).

Hormone Secretion. Both insulin and somatostatin were released into the culture medium by RIN-r cells. The amounts secreted varied during the first five passages, with a maximum for insulin of 100 μU/10^6 cells per 24 hr. Levels then stabilized in the range of 25–50 μU/10^6 cells per 24 hr. Somatostatin detected in medium from early passages of the parent cells was in the range of 170–1300 pg/ml (cell counts not available).

Supernatant fluids of RIN-m cultures were also assayed for insulin activity at several different passages. Initially, sublines maintained in DMEM and RPMI-1640 media released relatively high levels of insulin (Fig. 6). With time, the levels fell, and in DMEM insulin could not be detected after 190 days. In RPMI-1640 medium insulin secretion levels dropped during the first 100 days. Subsequently they fluctuated widely, but eventually settled between 150 and 250 μU/10^6 cells per 24 hr. RIN-m cells maintained in RPMI-1640 medium have continued to secrete insulin for more than 400 days. Somatostatin was detected in the supernatant fluids of all RIN-m sublines during both early and late culture life. In contrast to insulin, somatostatin levels did not fluctuate widely with culture time or type of medium, and ranged from 3150 to 8700 pg/10^6 cells per 24 hr.

Analysis of hormone secretion by clones of RIN-r and RIN-m

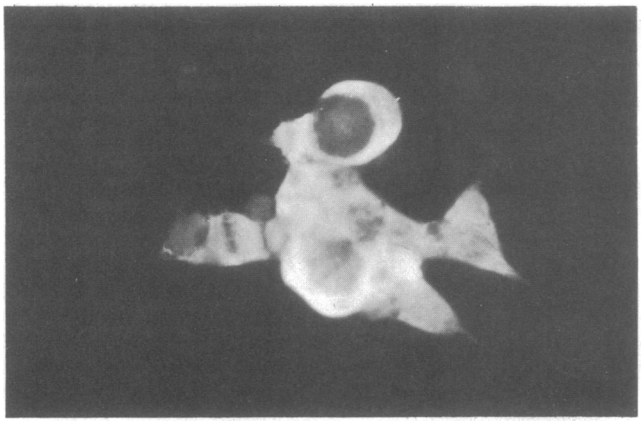

FIG. 5. Cytoplasmic fluorescence induced in RIN-m cells after exposure to formaldehyde vapor. (×950.)

cell lines revealed a wide range of insulin secretion, from undetectable to relatively high (Table 1). Preliminary data indicate that at least some clones secrete somatostatin.

The presence of glucagon in the supernatant fluids of the parent lines and clones could not be unequivocally demonstrated, because the values obtained were near the lower limit of detectability of the assay (15 pg/ml of supernatant fluid).

DISCUSSION

The cultures described in this communication possess several interesting features. They are continuous, clonable, hormone-secreting cell lines of islet cell derivation. They contain hypodiploid epithelioid cells devoid of fibroblast contamination, which are tumorigenic in homologous rat hosts and in athymic *nude* mice.

The cell lines and some of the clones secrete both insulin and somatostatin, as does the transplantable tumor from which they were derived (6). While levels of somatostatin secretion by the parent cell lines have been fairly constant, insulin levels have fluctuated widely and have varied with culture time and type of growth medium. Insulin secretion by the clonal isolates also varied considerably. Variations in insulin secretion by the cultures may, in part, be due to the simultaneous secretion of somatostatin, an inhibitor of insulin secretion (19).

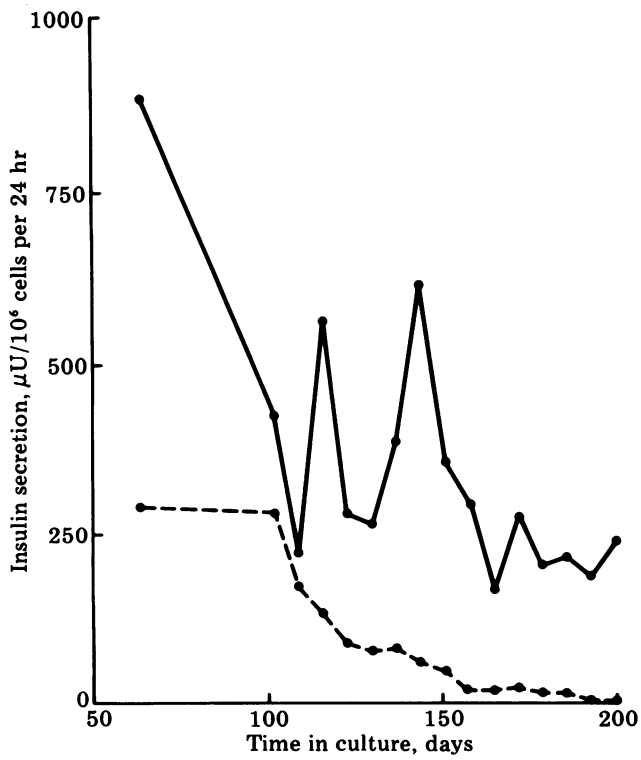

FIG. 6. Insulin secretion by RIN-m cells maintained in DMEM (●---●) or RPMI-1640 (●—●) medium.

Table 1. Insulin secretion by RIN cells and clones

Cell designation	Insulin secretion, μU/10⁶ cells per 24 hr
RIN-r, passage 20	22
Clone 1	4178
Clone 2	238
Clone 3	72
Clone 4	31
Clone 5	226
RIN-m, passage 6	280
Clone 1	2158
Clone 2	980
Clone 3	84
Clone 4	0
Clone 5	146

RIN-r cells, grown in medium 199, were cloned after 20 passages, and RIN-m cells, grown in DMEM, after 6 passages. Cells (10–100) were seeded into culture dishes (RIN-r) or microtiter plates (RIN-m). Individual colonies in dishes were isolated with penicylinders. Cells were harvested from microtiter wells in which only single colonies were observed. Colonies were grown to mass culture and tested for insulin secretion after three passages.

The parent cultures were established by two investigative groups using different approaches. Sato and coworkers (20) demonstrated that functioning endocrine tumor cultures are established more readily by cycling cultured cells through suitable animal recipients. The RIN-r cell line was established after one such cycle. In contrast, the RIN-m cell line was established directly from a fourth passage tumor heterotransplanted into athymic *nude* mice. Attempts to establish cell lines from earlier passages failed, but continuous cell lines have been established from subsequent passages. Presumably some as yet unknown alteration occurred in the tumor during serial heterotransplantation. The *nude* mouse tumors consisted of rat islet cells supported by stromal cells of mouse origin. Isoenzyme studies confirmed the rat origin of RIN-m cells. Human tumors heterotransplanted into *nude* mice are frequently contaminated with murine leukemia virus strains (21), but RIN-m cells are apparently free of the virus.

The cell lines have biochemical, functional, and morphological features that include them in the APUD cell series of Pearse (22). APUD cells, and the tumors derived from them, belong to a miscellaneous collection of cells present in several organ systems, having in common the prime function of polypeptide hormone or amine production, and possessing mechanisms for the selective uptake, storage, and decarboxylation of amine precursors. RIN cultures secrete polypeptide hormones, have high levels of the key APUD cell enzyme L-dopa decarboxylase, and fluoresce after exposure to formaldehyde vapor (indicating the presence of fluorogenic amines). The cells contain membrane-bound secretory granules (unpublished data, obtained in collaboration with A. Like). Although Pearse postulated that APUD cells originate from the neural crest (22), the islet cells are likely of endodermal origin (23).

The RIN cell lines offer important models to study the

mechanisms and control of insulin and somatostatin secretion. They may also be used to identify hormone receptors and antigens on their surfaces and to determine the nutritional requirements for hormone secretion in defined media. If hormone secretion is expressed dominantly in interspecies hybrid cells, they may be used to identify the genes regulating hormone synthesis. Such studies will be aided by the identification of clonal cultures secreting single hormones.

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