An Established RIII Mouse Mammary Tumor Cell Line; Kinetics of Mammary Tumor Virus (MTV) Production[1] (36119)

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Continuous production of the mouse mammary tumor virus (MTV) in tissue culture provides clean material for molecular studies and the eventual preparation of vaccines. To date, only one cell line was known to produce the mammary tumor virus continuously; it was derived from a spontaneous mammary tumor in a (C57BL × Af) F_1 hybrid mouse which theoretically should be MTV-free (1, 2). There is reason to believe that the virus produced is residual from the Af mouse and therefore is a foster-virus less potent than viruses from RIII or C3H mice.

In several attempts to establish a mammary tumor cell line from the RIII mouse or other strains, the production of B particles (MTV) decreased to zero in subsequent passages (3–5).

Recently, Dmochowski *et al.* (6) have listed a number of mouse mammary tumor cell lines carried in their laboratory among which are a RIII-MT (D-W) and a (C57BL × Af) F_1 Sykes subline. All of them except the latter have shown the production of a C-type virus. Even though no C particles were seen in cultures of the Sykes subline these authors have reported the presence of intracysternal A's which are morphologically related to C particles. About 2.5 years ago we isolated a cell line from an RIII mouse mammary tumor which has shown the exclusive release of B-type particles.

Even though a relatively large number of budding MTV particles was seen by electron microscopy at the surface membrane of RIII-MT cells, very few mature virions were found in high-speed pellets of the culture supernatants. The present report describes the origins of the RIII-MT cells and various attempts to liberate mature MTV from the cell membrane to establish a practical harvest.

Materials and Methods. The RIII-MT cells were isolated in Dec. 1968 from a soft spontaneous mammary tumor which occurred in a 10-month-old RIII mouse. After dissociation of the minced tumor in a calcium–magnesium-free saline with 0.25% trypsin, the cells were distributed in T30 Falcon plastic culture flasks in 2×10^6 aliquots. The medium, renewed three times a week, was Eagle's MEM (7) enriched with 10% fetal calf serum, 2.5% lactalbumin hydrolysate and 20 μg of insulin/ml. Penicillin and neomycin were added in the concentration of 500 units and 250 μg/ml, respectively.

For electron microscopy, the low-speed centrifugation pellet of freshly trypsinized cells was fixed in 1% buffered osmium tetroxide and embedded in Epon. Thin sections were observed in an A.E.I. model EM6B electron microscope.

A quantitative estimation of the number of virus producing cells was obtained from whole cell mount electron microscope preparations (8). The results obtained by this method have been found to correlate quite well with the presence of MTV antigen as detected by cell-membrane immunofluorescence.

Immunofluorescence was done on suspensions of live cells, using the indirect method

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of Klein *et al.* (9). Antisera produced in the rabbit against B particles and in the rat against Gross leukemia virus were used comparatively (10).

Release of mature particles in the tissue culture supernatant was evaluated from high-speed pellets of cell-free culture fluids. After preliminary low-speed centrifugation to eliminate large particulate matter, the supernatants were further clarified by centrifugation at 5000 rpm for 10 min in a Spinco SW 25.1 rotor. A volume of 12 ml clear supernatant then was spun at 32,000 rpm for 1 hr in a SW 39 rotor. The high-speed pellet (HSP) was resuspended in 3 drops of isotonic phosphate buffered saline (PBS) and 1 drop of this suspension was placed on a 300 mesh electron microscope grid precoated with a carbon film. Following negative staining with sodium phosphotungstate the grids were examined in a Siemens Elmiskop 1A electron microscope. Virus particles were counted in 10 grid-squares. HSP scale ratings were set as follows: 1+ for 1 virus particle/square; 2+ for an average of 2 to 5 particles/square; 3+ for 6 to 10 particles; and 4+ for 11 or more particles.

Bioassays consisted in the inoculation of 0.1 ml of tissue culture supernatants into the peritoneal cavity of 6-week-old female C57BL mice. Milk taken at the third lactation was tested by the microimmunodiffusion technique of Charney *et al.* (11) for the presence of MTV antigens.

Results. The primary cells obtained from the dissociation of the original RIII mammary tumor were epithelial and became confluent in about 10 days. Following a first subculture, only a few colonies settled down and remained dormant for the next 5 months. After 6 months, the cultures could be split in a 1:2 ratio every 2 weeks; from month 12 on, a 1:4 split ratio was done at 10 day intervals. The cells were epithelial and have retained this morphology (Fig 1).

Thin sections of the cultures prepared at 2–3 month intervals have consistently shown the presence of intracytoplasmic, doughnut-shaped A-type particles (Fig. 2), as well as of B-type particles budding from the cell membrane (Fig. 3). The B particles were characterized by their preformed core, the spikes appearing on the external membrane and the eccentric dense nucleoid of the mature virion (Fig. 4). B-type particles have been identified as the mammary tumor virus (12, 13) and cytoplasmic A particles are believed to be precursors of only B particles. C-type particles associated with the leukemia–sarcoma system have not been found; nor have intracisternal A particles been seen.

Infectivity of the culture supernatants was tested at 3 to 4 month intervals (Table I). MTV antigen was detected in the third lactation milk of 34 out of 42 C57BL female mice inoculated with the culture fluids. Hundredfold dilutions of the supernatants did not reduce the 80% ratio of infected mice; several of these animals have developed mammary tumors as could be predicted from earlier correlations of positive immunodiffusion with tumor incidence (11). None of them developed leukemia.

TABLE I. Infectivity of RIII-MT Culture Supernatants.[a]

Dilutions	Expt. 1	2	3	4	Total	%
10^{0}		10/10[b]	16/20	8/12	34/42	80
10^{-1}	4/4	11/12	16/19	9/12	40/47	85
10^{-2}	5/6	8/8	11/16	8/10	32/40	80

[a] Culture fluids filtered through a 0.45 μ Millipore membrane; 0.1 ml was injected intraperitoneally into 6–8-week-old C57BL mice.

[b] Number of mice infected per total number of mice inoculated.

In addition, inoculation of 4×10^{6} RIII-MT culture cells into newborn RIII mice produced large size nodules at the site of injection; histological sections have revealed a typical infiltrating carcinoma (Fig. 5). Evidence that the cultivated cells were a neoplastic epithelium producing the mammary tumor virus was therefore obtained.

Extensive budding of virus particles at the cell margin was also demonstrated in negatively stained whole cell mount preparations of RIII-MT cells (Fig. 6). Quantitative studies of whole cell mounts showed that 80% of the cells in the culture were producing virus 7 days after subculturing. This high

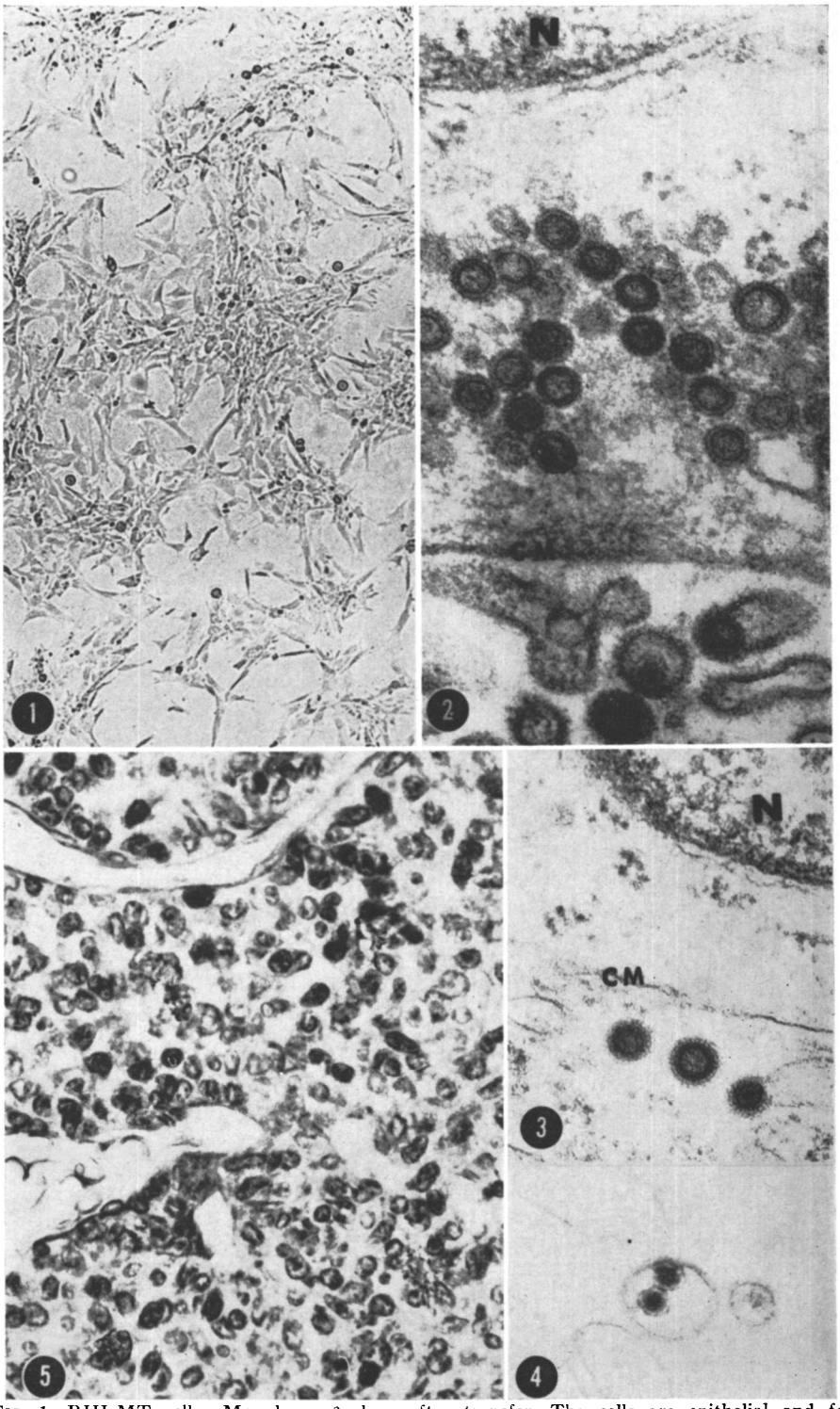

FIG. 1. RIII-MT cells: Monolayer 3 days after transfer. The cells are epithelial and form multilayered colonies; ×675.

FIG. 2. Thin section of RIII-MT cells showing intracytoplasmic A-type particles: (N) nucleus; (CM) cell membrane; ×67,500.

FIG. 3. Thin section of RIII-MT cells showing budding B particles: Note that the core of the particle is complete before separation from the cell membrane. The spikes outline the envelope of the virion. (N) nucleus; (CM) cell membrane; ×54,000.

FIG. 4. Mature, binucleated B particle in the intercellular space: Another B particle less intensely stained is beside it; ×54,000.

FIG. 5. Histological section through an 8-day-old nodule which developed in a newborn RIII mouse injected with 4×10^6 tissue culture RIII-MT cells. Hematoxylin–eosin stain; ×2700.

production was maintained up to about day 14, whereupon it gradually decreased. Immunofluorescence confirmed these results. Using an anti-B particle rabbit serum, about 50% of the RIII-MT cells were found positive 7 days after subculturing; this percentage declined thereafter. When positive, the cells presented at their equator a characteristic fluorescent ring indicating the presence of MTV antigen; the same antiserum did not react with MTV-free cells or with leukemia-infected cells. In the same manner a Gross leukemia virus antiserum gave positive fluorescence with leukemia virus-infected cells but not with the RIII-MT cells (Table II).

Contrary to the above findings, no virus was found in high-speed pellets of culture medium taken 9 to 10 days after subculturing; and only a 1+ count of virions was observed in such preparations taken after 12 days.

In various attempts to release the virus from the cells, neither insonation nor osmotic shock increased the yield of free virions. Freeze-thawing the cell monolayers three times in their culture medium or in distilled water did not liberate the virus. However, when the cells were first trypsinized, resuspended in their culture medium, freeze-thawed, then stored overnight at 4°, the HSP count was 3+.

TABLE II. Comparative Membrane Immunofluorescence of RIII-MT Cells with a Leukemia Virus Producing Cell Line (GTT).[a]

Cell line	NRbS	MTV-AS	NRtS	LV-AS
RIII-MT	—	+	—	—
GTT	—	—	—	+

[a] NRbS = normal rabbit serum; MTV-AS = rabbit anti-MTV serum; NRtS = normal rat serum; and LV-AS = rat antileukemia virus serum.

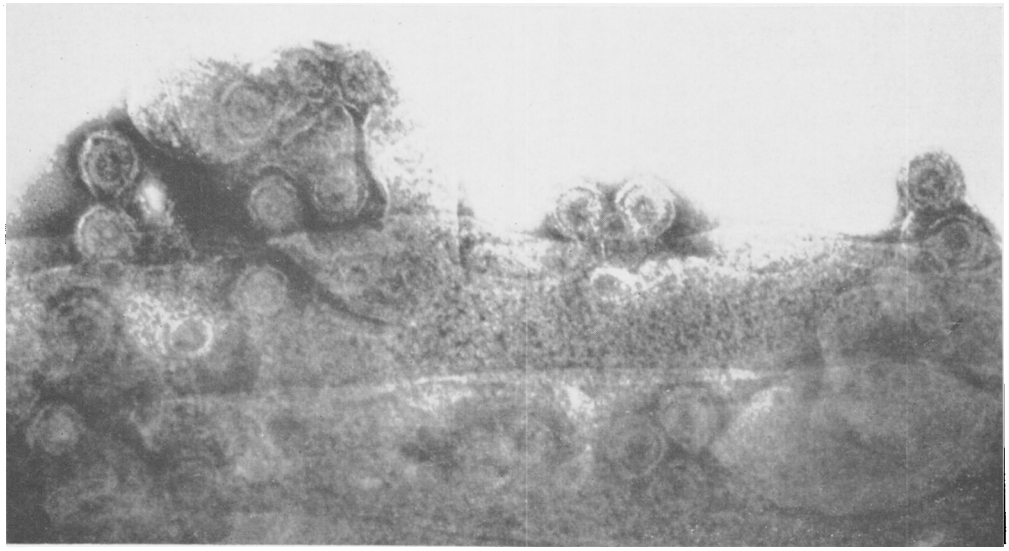

FIG. 6. Negatively stained whole cell mount of an RIII-MT cell showing numerous virions budding at the cell margin. ×80,500.

From the above results, it appeared that a maximum yield of virions could be obtained after refrigeration. RIII-MT cells were therefore grown for 12 days to obtain the highest percentage of virus producing cells, then stored at 4°. Aliquots of 12 ml of supernatant were tested for virus particles after 3, 6, 11, and 14 days storage. As shown in Table III,

TABLE III. Effect of Refrigeration and Cell Dispersion on the Release of B Particles from RIII-MT Cells.

Particle count as in Table I.

Time postrefrigeration (days)	Refrigeration of	
	Intact monolayers	Dispersed cells
0	<1+	<1+
3	1+	
6	2+	
11		3+
14		3+

a slight increase in the number of virions was noted after 6 days. The cell sheets were then broken up by scraping and pipetting; the amount of virus found on days 11 and 14 of storage then rose to 3+, suggesting a beneficial effect of cell dispersion.

In a second experiment, HSP of the culture supernatant at the end of 12 days and prior to refrigeration was less than 1+. The cells were then dispersed by trypsin, resuspended in their culture medium, and stored at 4° for 7 days. HSP of the cell-free supernatant was then 3+, thus confirming our previous results. The 7-day-old refrigerated supernatants tested by inoculating aliquots of 0.1 ml in the peritoneal cavity of C57BL weanling female mice showed infective activity by Charney's test in 54 out of 57 mice.

Discussion. It appeared evident that the RIII-MT cell line was a good producer of MTV but that very few mature virions were released into the extracellular fluid. Since the virus particles, which accumulated at the cell surface, remained attached in an apparent halt of their evolution towards maturity, it was likely that the pinching mechanism which usually liberates the virions was somewhat defective. This was overcome by two factors: (a) the separation of cells from each other either mechanically or enzymatically; (b) refrigeration for several days. Apparently the pinching mechanism which released the virus was triggered by these physical influences. Storage in the cold for a least 7 days did not alter the infectivity of the released virions as demonstrated by a preliminary bioassay. Titrations of the bioactivity of MTV harvested from RIII-MT cells are in progress.

Summary. A cell line derived from an RIII mouse mammary tumor has been successfully established. This cell line, RIII-MT, is epithelial and after nearly 3 years cultivation has been shedding a membrane-budding virus continuously. This virus was shown to be the mammary tumor virus by electron microscopy, immunofluorescence and bioassays. No cross-reaction was found with the leukemia–sarcoma system.

The MTV produced at the cell surface of RIII-MT cells was not spontaneously released. Harvest of the virions was obtained first by cell dispersion, then by storage for 7 days at 4°. After refrigeration, the virus retained full infectivity.

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