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A HUMAN BREAST TUMOR CELL LINE (BT-474) THAT SUPPORTS MOUSE MAMMARY TUMOR VIRUS REPLICATION

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SUMMARY

A human breast tumor cell line BT-474 derived from an invasive ductal carcinoma was experimentally infected in vitro with a mouse mammary tumor virus from the RIII strain (RIII-MuMTV). The virus that replicated in the human cells was characterized as a mouse virus by immunofluorescence, electron microscopy and the presence of a specific RNA-directed DNA polymerase. The cells themselves were human as per the karyotype and isoenzyme migration patterns. It is concluded that human cells are susceptible to the mouse mammary tumor virus and can, eventually, support its replication.

Key words: human breast; mammary tumor virus; experimental infection.

INTRODUCTION

Ever since the initial discovery in 1936 (1) of a viral agent involved in mouse mammary carcinogenesis, a tremendous effort has been aimed at the demonstration of a similar agent in human breast tumors. The impressive amount of suggestive evidence accumulated over the past 10 years, however, is not sufficient to substantiate this concept. Even though rare viral particles morphologically identical to that of the mouse (2,3) and a possible RNA-directed DNA polymerase (4,5) have been detected in the milk of women, no valid correlation between their presence and the neoplastic state was ever established (6,7). Serological cross-reactions between human and mouse tumors have been the object of many tantalizing observations (8–10). For the moment it seems that such cross-reactions might result from the presence, at the surface of some human tumor cells, of a protein component sharing a few physical properties with a major glycoprotein of MuMTV (11).

Whether this component is the expression of a small part of the MuMTV genome integrated into the cell's genetic material for several generations, or an entirely different product unrelated to the virus, is not known. However, despite an acute awareness of viral possibilities and increased scrutiny of human material in several laboratories, no virus particle comparable to that of the mouse has ever been observed budding from the surface of human breast tumor cells. Out of the 117 solid breast carcinomas that we processed, 52 have grown in tissue culture for periods of 4 to 6 months, but none has shown evidence of virus budding by electron microscopy. By using isolation procedures described elsewhere in detail (12), well characterized epithelial cell lines were derived from some of these breast specimens (13); because of a relatively fast growth rate, one of the cell lines, BT-474, was specifically selected to test the susceptibility of human cells to infection by RIII-MuMTV.

Experimental infection of cells by the mouse mammary tumor virus in tissue culture is a recent advance initiated in our laboratory (14,15). With the use of similar techniques we have obtained evidence that BT-474 cells are a compatible host for the mouse mammary tumor virus. A description of this evidence and speculations about its possible meaning for the human disease, therefore, are presented.

MATERIALS AND METHODS

Cells. The BT-474 cells were derived 2 years ago from an invasive ductal breast carcinoma in a white 60-year-old patient (13). The cells used in

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this study were taken from a frozen stock stored in liquid nitrogen and tested at the time of freezing for eventual mycoplasma contaminations. To detect the presence of mycoplasma, scraped cells and their supernate were distributed in aliquot portions into tubes containing brain-heart infusion broth or spread out on top of an agar medium. After a proper time of incubation in aerobic and anaerobic conditions, following the techniques described by McGarrity (16), no mycoplasma were found. Morphologically these cells are epithelial; the presence of tight junctions, gap junctions, desmosomes, tonofibrils and abundance of microvilli at the cell membrane (13) are all well accepted characteristics of their epithelial nature. They grow uniformly, doubling their population in 72 hr in a complete culture medium composed of 10% fetal bovine serum (FBS) in RPMI-1640 (Flow Laboratories, Bethesda, Maryland) and supplemented with 0.23 IU per ml insulin (Schwarz/Mann, Orangeburg, New York). To facilitate the detection of accidental bacterial infections, antibiotics are not used. Morphologically, the chromosomes of the BT-474 cell line are human; their modal number was estimated at 55 and Giemsa banding did not reveal the presence of HeLa markers.

Inoculation of 4×10^6 cells into nude mice initiated tumors and therefore confirmed their neoplastic nature (13). *Inoculation* of BT-474 cells with RIII-MuMTV was done by procedures already described (14). Briefly, cultures enzymatically dissociated (0.05% EDTA-trypsin; GIBCO, Grand Island, New York) to yield single cell suspensions were resuspended in viral and nonviral inoculum at the rate of 10^6 cells per ml. The viral inoculum itself was prepared from a 4-ml volume of fresh milk collected and pooled from RIII nursing mice. After skimming, the virus was pelleted by high-speed centrifugation and purified through a 25% Ficoll gradient (Pharmacia, Uppsala, Sweden). After 1-hr centrifugation at 32,000 rpm in a Spinco 50.1 rotor, the band that formed at the 1.052 g per ml density level was removed and reconstituted to the original 4-ml volume in Hanks'-Eagle medium (Flow Laboratories, McLean, Virginia). Polybrene (Aldrich Chemical Co., Milwaukee, Wisconsin) was added in sufficient quantity to provide a final concentration of 4 μg per ml. The nonviral inoculum was made of Hanks'-Eagle medium containing comparable amounts of Ficoll and polybrene; both were sterilized by filtration through a 0.45-μm Millipore membrane.

The cells suspended in their respective inocula were incubated for 1 hr at 37° C in a water bath after which each ml of the suspension was diluted 5-fold in complete culture medium and distributed into 25-cm^2 culture flasks (Falcon, Oxnard, California).

At 24 hr, and 4 and 8 days after inoculation, the medium was withdrawn, and the cells were extensively washed in Hanks'-Eagle solution and refed with fresh complete medium. On day 10 the cells were tested for the presence of MuMTV by membrane immunofluorescence (IF). Forty-eight hr before the test, the culture medium was supplemented with 10^{-5} M dexamethasone (Sigma Chemical Co., St. Louis, Missouri), a synthetic steroid noted to enhance virus production (17). The indirect method of immunofluorescence previously described (18) was used. Antisera were

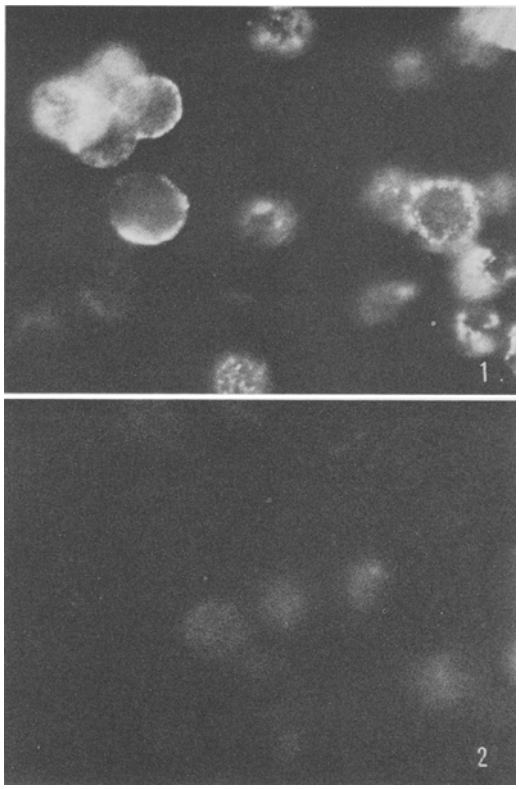

FIG. 1. BT-474 Cells showing positive membrane immunofluorescence after exposure to RIII-MuMTV antiserum. The photograph was taken 1 year after initial inoculation. ×1020.

FIG. 2. BT-474 Control cells exposed to the same RIII-MuMTV antiserum. The cells had been exposed to a sham inoculum without virus; no fluorescence is observed. ×1020.

TABLE 1

IMMUNOFLUORESCENCE OF HUMAN BREAST TUMOR CELLS INOCULATED WITH RIII MuMTV

Cells	Time	MuMTV[a]		MLV[b]		NRS[c]	NGS[d]
		Virions	Gp55	RLV	GLV		
	wk						
BT-474 i	1½	+	+ (8%)	—	—	—	—
	4	+	+ (60%)	—	—	—	—
	52	+	+ (60%)	—	—	—	—
BT-474 c	1½	—	—	—	—	—	—
	4	—	—	—	—	—	—
	52	—	—	—	—	—	—

[a] Sera used for the reactions: MuMTV. Virions, anti-mouse mammary tumor virions; Gp55, anti-glycoprotein 55 of the RIII-MTV.
[b] MLV, Mouse leukemia virus; RLV, Rauscher leukemia virus; GLV, Gross leukemia virus.
[c] NRS, Normal rabbit serum.
[d] NGS, Normal goat serum.

prepared in the goat against whole virions purified from RIII milk and against gp55, a specific viral membrane protein extracted from RIII virions; their titer estimated by Ouchterloney immunodiffusion was 1:40 and 1:60, respectively. Two other antisera prepared in rabbits against Gross and Rauscher leukemia viruses were obtained from NIH Resource Center; both reacted with a mouse testicular cell line (GTT) characterized as a high leukemia virus producer by eliciting membrane fluorescence in 95% to 100% of the cells. Normal goat and normal rabbit sera, absorbed with human embryonic cells, also were used. Labeling was done with corresponding fluorescein conjugates of commercial source (GIBCO, Grand Island, New York). All sera were used at a 1:10 dilution in Hanks' saline.

Six months after inoculation, the infected cells and their controls were prepared for electron microscopy according to the routine procedures of our laboratory (18). Briefly, the cells were scraped, fixed in 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated and embedded in Epon-araldite. Thin sections obtained with a diamond knife were sequentially stained with uranylacetate and lead citrate and examined in a JEOL-JEM electron microscope.

One year after inoculation, assays to detect an RNA-directed DNA polymerase (RDDP) were conducted on 100X concentrates of culture supernates using synthetic template/primers: oligo (dG)-poly(rC).

RESULTS

Morphology. After inoculation with RIII-MuMTV, experimental and control cultures assumed the same morphology as the BT-474 stock cells; furthermore, no perceptible differences in the rate of growth or metabolism of the cultures permitted at this time to distinguish the infected cells from the sham-inoculated controls.

Immunofluorescence (IF). Evidence of virus infection was first observed 10 days after inoculation. As shown in Fig. 1, single cell suspensions from MuMTV-infected cultures presented, after exposure to the specific antiserum, a number of cells with a bright continuous fluorescence of their outer membrane. These were not seen in suspensions from control cultures (Fig. 2). The specificity of the reaction was further confirmed by the experiments summarized in Table 1; infected cells did not react with antisera against Gross or Rauscher leukemia viruses nor with normal goat or rabbit sera. Cell suspensions from stock and control cultures remained negative in all cases. About 5% to 8% of the infected cell population was positive 10 days after inoculation. By maintaining dexamethasone in the culture medium the number of positive cells increased to 60% and then remained constant. These results were qualitative; there was in particular a 4- to 6-fold difference between the percentage of positive cells tested with whole MuMTV antiserum and the percentage of positive cells tested with anti-gp55 serum. The absence of fluorescence in preparations of noninfected cells gave conclusive evidence of the specificity and reliability of the reaction.

Electron microscopy (EM). The epithelial features of the cells, such as the presence of desmosomes, tight junctions, microvilli, tonofibrils and large indented nuclei, were found comparable to those of BT-474 stock cells. However, for the first

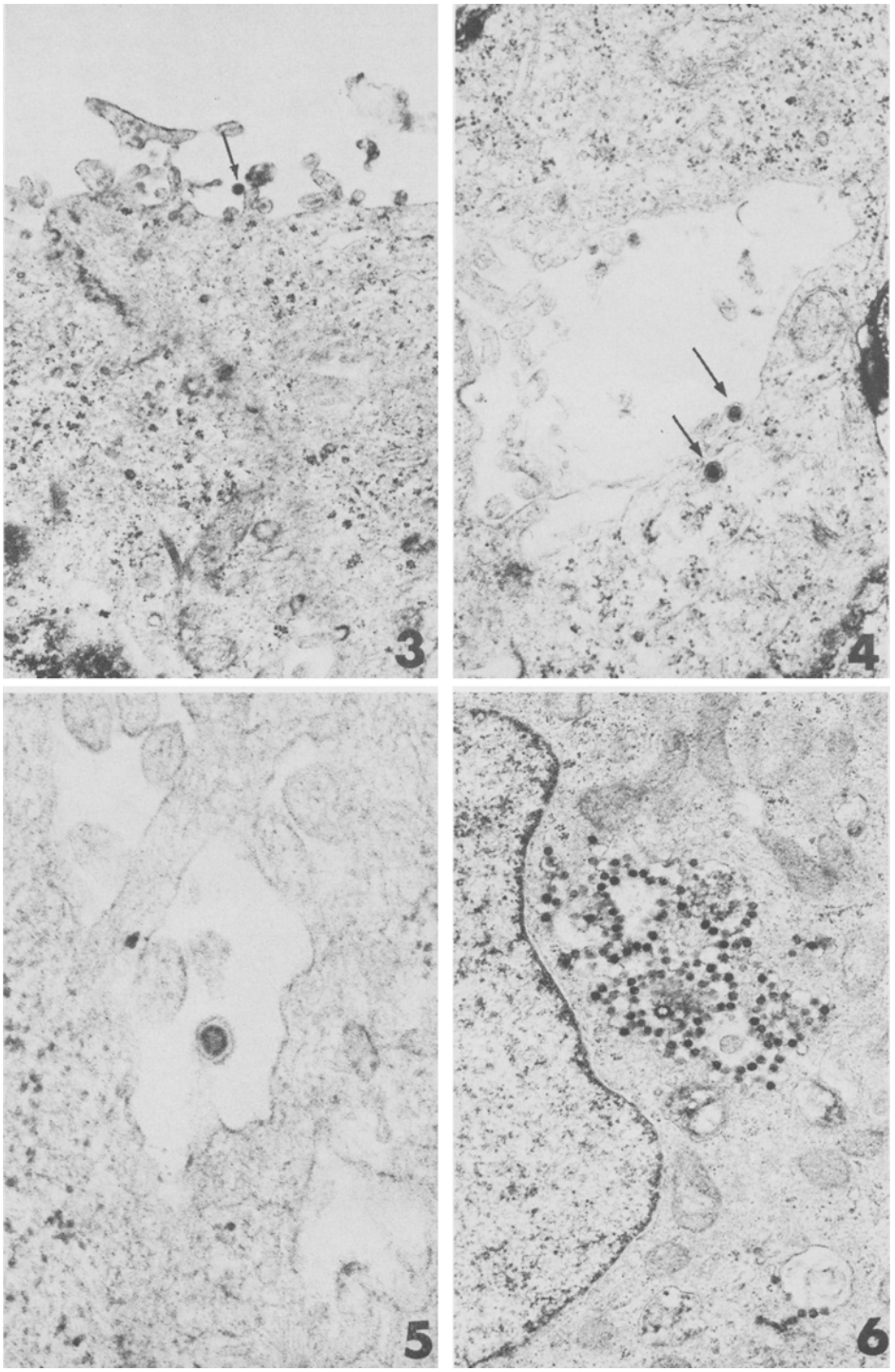

FIG. 3. Electron micrograph of a BT-474 cell margin showing budding of an immature virus particle (*arrow*). ×18,700.

FIG. 4. *Arrows* point to more virus particles budding from an infected BT-474 cell. ×31,800.

FIG. 5. Higher magnification of a B particle produced at the tip of a microvillus. ×51,660.

FIG. 6. Cluster of introcytoplasmic A particles in a BT-474 cell. ×18,700.

TABLE 2

RDDP ASSAYS OF CONCENTRATES OF TISSUE CULTURE SUPERNATES

Template/Primer	Cation	pmol [³H]dGMP Incorp./30 min[a]
MuMTV-infected		
oligo(dG)·poly(rCm)	Mn++	1.23
oligo(dG)·poly(rC)	Mg++	2.54
oligo(dG)·poly(rC)	Mn++	0.12
oligo(dG)	Mg++	0.01
oligo(dG)	Mn++	0.01
Uninfected control		
oligo(dG)·poly(rCm)	Mn++	0.01
oligo(dG)·poly(rC)	Mg++	0.01
oligo(dG)·poly(rC)	Mn++	0.01

[a] Expressed as trichloroacetic-acid-precipitable counts. The method of assay was described previously (5), and all results were corrected for label trapping and/or endogenous enzymatic activity.

time, small amounts of viral particles were observed budding from the surface membrane of the inoculated cells (Figs. 3–5). Even though the characteristic spikes that are a hallmark of the external coat of MuMTV were not clearly defined, the fact that the viral nucleoids were completely formed before the time of budding (Figs. 4,5) identified them as B rather than C particles; this conclusion was clearly supported by the IF results. Furthermore, clusters of intracytoplasmic A particles also were observed in many cells (Fig. 6). These structures, described as possible precursors of B particles (19), were numerous in the infected cells, but none were formed in the controls.

RNA-directed DNA polymerase (RDDP). Assays to detect RDDP in supernates of the infected and control cultures revealed the presence of a specific RDDP reaction in the spent medium of the inoculated cells. As shown in Table 2, no measurable enzymatic activity was observed in control culture supernates. In contrast, supernates from MuMTV-infected human cells possessed significant polymerase activities with oligo (dG) poly(rCm) or oligo (dG) poly (rC). In addition, the obvious Mg^{++} cation preference in the presence of oligo (dG) poly (rC) is typical of type-B virions (5), corroborating electron microscopic and immunofluorescence data reported in this study. Finally, no reaction was observed in the presence of only the primer, oligo(dG); therefore the results obtained were not due to a terminal transferase reaction.

Virus infectivity. The virus produced by the human cells was infectious to the mouse; 1 year after infection with MuMTV, BT-474 cells injected intraperitoneally into 8-week-old C57Bl mice at the rate of 4×10^6 cells per mouse induced mammary tumors in 3 out of 5 mice; none occurred in the noninoculated controls. The cells did not grow in the peritoneal cavity and were apparently rejected as human cells; the virus, however, elicited the mammary tumors.

Evidence for human cells. The replication of a mouse virus in human cells was unusual enough to stimulate investigation of whether accidental contamination by mouse cells had occurred. This was done by karyotyping and isoenzyme analysis. The karyotype of the infected cells was done 1 year after inoculation. Twenty-four-hr replicate cultures were exposed to colcemid for 1 hr, trypsinized, treated in hypotonic solutions, air-dried and stained with Giemsa. The trypsin-Giemsa staining efficiently detects chromosome breaks, sister chromatid exchanges or the presence of markers belonging to other cell lines (20). In this case the morphology of the chromosomes was unquestionably human; no chromosome related to mouse or to the genotype of other species was found. Furthermore, the modal chromosome number of 60 was comparable to that of the BT-474 stock line.

The isoenzyme analysis done by gel electrophoresis as described by Gartler and Farber (21) confirmed these results. Extracts from infected and control BT-474 cells were tested for the electrophoretic migration of G-6-PD, LDH, MDH and 6-GPD isoenzymes and simultaneously compared with the migration activity of the same enzymes from mouse, rat and hamster cells, and various human cell lines. It was found that migration of G-6-PD produced a fast-moving B band, which clearly excluded contamination by HeLa cells; all other enzymes presented a migration pattern distinct from those of mouse, rat and hamster cells but comparable to that of the human lines.

DISCUSSION

Evidence was obtained that human cells are indeed susceptible to the mouse mammary tumor virus. The highly specific immunofluorescence demonstrated 1 year past inoculation, the budding of virus particles from the cell membrane as observed by electron microscopy in the same time sequence, and the simultaneous presence of a specific viral reverse transcriptase in the culture

supernates are unquestionable proof that the mouse mammary tumor virus replicated in these human cells. The fact that only 60% of the BT-474 cells remain positive after 52 weeks even under stimulation with dexamethasone might be related to the low replicative activity of the cells. It is known that viral infectivity is most efficient when cells are in log-phase replication; the low growing power of human breast tumor cells would, therefore, limit the initial percentage of infected cells. Since the spread of infection from cell to cell is negligible, the later increase of positive cells would greatly depend on their rate of replication. Experimental infection of BT-474 cells was successfully repeated with another human carcinoma cell line (BT-483), but, as in the mouse, fibroblasts (IMR-90) have been nonpermissive to MuMTV replication.

In terms of human disease—could these results imply that the mouse mammary virus is eventually at the origin of breast cancer? In this particular case it appears unlikely because the susceptible cells, already neoplastic when the experiment was initiated, did not show detectable cross-reactivity with mouse or MuMTV antisera. No virus was originally demonstrable by the techniques available; nevertheless, experimental infection has conclusively shown that human cells can act as perfectly compatible hosts to the mammary tumor virus. Is it possible, then, that when sera from breast cancer patients cross-react by immunofluorescence with mouse mammary tumor cells or when the leukocyte migration from these patients is inhibited by MuMTV, the virus might be the initial cause? One should keep in mind that even in such instances the presence of viral particles associated with the original tumor has not been observed. One might argue, however, that if the viral infection is a long-past event, the existence of only partial sequences of complementary viral DNA integrated into the cell genome might not be sufficient to initiate the synthesis of a complete virion; instead it might code for various viral proteins and this would account for the various humoral and cell-mediated cross-reactions reported in the literature. A recent report by Yang, McGrath and Furmanski (22) appears to support this view; however, the existence on MCF7 cells of a surface glycoprotein cross-reacting with a major glycoprotein of MuMTV does not necessarily indicate the presence of a whole viral genome. Budding viral particles have never been demonstrated in MCF-7 cells; and, furthermore, we did not find a comparative reaction in six breast tumor lines derived from solid carcinomas. Only techniques of DNA-DNA hybridization might be able to answer the question of partial viral sequences in human tumor cells; these are presently under investigation.

REFERENCES

1. Bittner, J. J. 1936. Some possible effects of nursing on the mammary gland tumor incidence of mice. Science 84: 162.
2. Seman, G., B. Myers, W. C. Williams, H. S. Gallager, and L. Dmochowski. 1969. Studies on the relationship of viruses to the origin of human breast cancer. II. Virus-like particles in human breast tumors. Tex. Rep. Biol. Med. 27: 839-866.
3. Moore, D. H., J. Charney, B. Kramarsky, E. Y. Lasfargues, N. H. Sarkar, M. J. Brennan, J. H. Burrows, S. M. Sirsat, J. C. Paymaster, and A. B. Vaidya. 1971. Search for a human breast cancer virus. Nature 229: 611-615.
4. Shlom, J., and S. Spiegelman. 1971. Simultaneous detection of the reverse transcriptase and high molecular weight RNA unique to the oncogenic RNA viruses 1971. Science 174: 840-843.
5. Dion, A. S., A. B. Vaidya, and G. S. Fout. 1974. Cation preferences for poly (rC) oligo (dG)-directed DNA synthesis by RNA tumor viruses and human milk particulates. Cancer Res. 34: 3509-3515.
6. Sarkar, N. H., and D. H. Moore. 1972. On the possibility of a human breast cancer virus. Nature 236: 103-106.
7. Vaidya, A. B. 1973. Molecular biology of human milk. Science 180: 776-779.
8. Muller, M., P. C. Hageman, and J. S. Daams. 1972. On antigens in human breast cancer sera related to the murine mammary tumor virus. Nature (London) New Biol. 237: 116-117.
9. Priori, E. S., D. E. Anderson, W. C. Williams, and L. Dmochowski. 1972. Immunological studies on human breast carcinoma and mouse mammary tumors. J. Nat. Cancer Inst. 48: 1131-1135.
10. Mesa-Tejeda, R., I. Keydar, M. Ramanarayanan, T. Ohno, C. Fenoglio, and S. Spiegelman. 1978. Detection in human breast carcinomas of an antigen immunologically related to a group-specific antigen of mouse mammary tumor virus. Proc. Nat. Acad. Sci. U.S.A. 75: 15-29, 1533.
11. Black, M. M., R. E. Zachrau, B. Shore, A. S. Dion, and H. P. Leis. 1978. Cellular immunity to autologous breast cancer and RIII-murine mammary tumor virus preparations. Cancer Res. 38: 2068-2076.
12. Lasfargues, E. Y. 1975. New approaches to the cultivation of human breast carcinomas. In: J. Fogh

(Ed.), *Human Tumor Cells* in Vitro. Plenum Publishing Corp., New York, pp. 51–77.

13. Lasfargues, E. Y., W. G. Coutinho, and E. S. Redfield. 1978. Isolation of two human tumor epithelial cell lines from solid breast carcinomas. J. Nat. Cancer Inst. 61: 967–978.
14. Lasfargues, E. Y., J. C. Lasfargues, A. S. Dion, A. E. Greene, and D. H. Moore. 1976. Experimental infection of a cat kidney cell line with the mouse mammary tumor virus. Cancer Res. 36: 67–72.
15. Lasfargues, E. Y., A. B. Vaidya, J. C. Lasfargues, and D. H. Moore. 1976. In vitro susceptibility of mink lung cells to the mouse mammary tumor virus. J. Nat. Cancer Inst. 57: 447–449.
16. McGarrity, G. J. 1975. Detection of mycoplasma in cell cultures. Procedure 75317. TCA Manual 1: 113–116.
17. Parks, W. P., Scolnick, E. M., and E. H. Kozikowski. 1974. Dexamethasone stimulation of murine mammary tumor virus expression. A tissue culture source of virus. Science 184: 158–160.
18. Lasfargues, E. Y., and J. B. Sheffield. 1976. Infectivity and oncogenicity of the mouse mammary tumor virus in tissue culture. J. Microbiol. Biol. Cell. 16: 29–34.
19. Tanaka, H. 1977. Precursor-product relationship between nonglycosylated polypeptides of A and B particles of mouse mammary tumor virus. Virology 76: 835–850.
20. Nelson-Rees, W. A., R. R. Flandermeyer, and P. K. Hawthorne. 1974. Banded marker chromosomes as indicators of intraspecies cellular contamination. Science 184: 1093–1096.
21. Gartler, S. M., and R. A. Farber. 1973. Biochemical identification of cells in culture. A. Human cell lines by enzyme polymorphism. In: P. F. Kruse, and M. K. Patterson (Eds.), *Tissue Culture Methods and Applications.* Academic Press, New York, pp. 797–804.
22. Yang, N. S., C. M. McGrath, and P. Furmanski. 1978. Presence of a mouse mammary tumor virus-related antigen in human breast carcinoma cells and its absence from normal epithelial cells. J. Nat. Cancer Inst. 61: 1205–1208.

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