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Cell Biology

Epithelial mouse mammary cell line exhibiting normal morphogenesis *in vivo* and functional differentiation *in vitro*

(cell culture/duct morphogenesis/casein synthesis/keratin expression)

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ABSTRACT **An epithelial cell line, designated COMMA-1D, was derived from mammary tissue of BALB/c mice in the middle of pregnancy. This line, in continuous cell culture for 12 months, exhibits several characteristics distinctive of normal mammary epithelial cells, including induction of casein synthesis *in vitro* and normal duct morphogenesis in the cleared mammary fat pads of syngeneic mice. The cells also form domes in high density culture and are positive for keratin intermediate filaments by indirect immunofluorescence. COMMA-1D cells have a near diploid number of chromosomes and do not grow in suspension culture or produce tumors in syngeneic hosts. This cell line should prove useful for studies examining the regulation of normal cellular differentiation of mammary cells as well as transformation of epithelial cells to the preneoplastic and neoplastic phenotypes.**

The murine mammary gland consists of a branching network of ducts that ramify throughout an adipose stroma. Upon pregnancy and lactation, the ductal cells differentiate into alveolar cells capable of milk secretion. During the pathogenesis of breast cancer, normal mammary epithelial cells can give rise to morphologically altered alveolar and ductal dysplasias. Both the alveolar dysplasias, termed preneoplastic hyperplastic alveolar nodules, and the ductal dysplasias, in turn, have a high probability of progressing to the neoplastic state (1).

Considerable effort has been directed toward the characterization of normal, preneoplastic, and neoplastic mammary cells and tissues *in vivo* and *in vitro* with respect to growth regulation, hormonal responsiveness, and biochemical properties (for a review, see ref. 2). As part of this effort, a significant amount of work has been directed toward the development of cell culture methodology for establishing cell lines of mammary epithelial origin. Although numerous tumorigenic mouse mammary cell lines of epithelial morphology have been described (3–6), only two nontumorigenic mouse mammary cell lines have been successfully established from nonneoplastic tissue (7–9). Neither of these lines produces any type of cellular outgrowth when transplanted in the mammary fat pads of syngeneic mice. Difficulties in maintaining the growth potential of cultured epithelial cells, coupled with complications due to overgrowth of fibroblastic cells, have dictated that studies on normal mouse mammary epithelial cells *in vitro* be confined to short-term primary cultures.

To date, no established epithelial cell line retaining mammary gland-specific morphological and functional differentiation *in vivo* has been reported. We describe herein the isolation and characterization of an epithelial cell line, COMMA-1D, established from normal mouse mammary gland tissue. This cell line exhibits several properties specific for normal mammary gland function.

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MATERIALS AND METHODS

Isolation of the COMMA-1D Cell Line. Mammary glands were collected from BALB/c mice in the middle of pregnancy. The tissues were minced finely and the cells were dispersed by collagenase treatment as described (10). The cells were plated in two 75-cm^2 cell culture flasks in the presence of Dulbecco's modified Eagle's medium (DME medium), supplemented with 10% fetal bovine serum and insulin (5 μg/ml). Cell cultures were incubated at 37°C in a water-saturated atmosphere containing 7.5% CO_2 in air. The cells were not passaged for several weeks. Thereafter, at irregular intervals over the following 5 months, overgrowing fibroblasts were removed from the epithelial monolayer by treatment with trypsin/EDTA (GIBCO) in phosphate-buffered saline. After 6 months, to facilitate the preferential growth of epithelial-like cells over fibroblastic cells, the culture was incubated in the presence of DME medium with 1% fetal bovine serum and and the following components (designated as "growth supplement"): insulin (5 μg/ml), transferrin (5 μg/ml), fibronectin (1 μg/ml), epidermal growth factor (10 ng/ml), endothelial cell growth factor (10 μg/ml), and sodium selenite (50 nM) (11). After several passages (subculturing the cells 1:2 at 2-wk intervals), a sufficient number of cells was produced for use in the experiments described below. Presently, the COMMA-1D cell line is subcultured weekly with 1:2 splits and is maintained in DME medium containing 1% fetal bovine serum, the growth supplement, 10 mM Hepes, and gentamycin sulfate (50 μg/ml).

Karyology. Exponentially growing cultures of COMMA-1D cells were incubated with colchicine (0.4 μg/ml) for 6 hr. Cells were removed from the substrate with trypsin/EDTA and treated with hypotonic medium for 10 min. Metaphase chromosome spreads were prepared as described by Moorhead (12). Chromosomes of at least 50 metaphase cells were counted in cells at passages 3 and 14.

Suspension Culture. The method of Seman (13) was used with slight modifications. Briefly, COMMA-1D cells were seeded at a concentration of 1.3×10^4 cells per cm^2 in 60-mm Petri dishes containing a 1.5% agarose layer previously equilibrated with DME medium containing 5% fetal bovine serum and the growth supplement. After 12–14 days, the cultures were examined for growth of cells as multicellular spheroids.

Collagen Gel Culture and Analysis of Casein Polypeptides. Cell suspensions taken from virgin mice and embedded into collagen gels can be induced to synthesize casein polypeptides by the addition of hormones to the growth medium (14, 15). COMMA-1D cells were embedded within a collagen (type I) matrix *in vitro* to determine inducibility of casein synthesis. Cells were seeded at a density of 1×10^5 cells per cm^2 in collagen gels according to the procedure of Yang *et al.* (16). The cells were allowed to grow for 3 wk at 37°C in growth medium containing DME medium supplemented with 50% horse serum, 2.5% fetal bovine serum, and insulin (10 μg/ml). The medium was then changed to induction me-

dium consisting of growth medium with prolactin (5 μg/ml), aldosterone (5 μg/ml), and hydrocortisone (1 μg/ml). The prolactin was kindly provided by the Pituitary Hormone Distribution Program of the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases. The cultures were allowed to incubate at 37°C for an additional week, during which time the medium was changed daily. For analysis of casein synthesis, the cells were dissociated from the collagen matrix by collagenase treatment and extracted with 8 M urea buffer (14). The proteins in the cell extract were separated on 10% polyacrylamide gels; then specific casein polypeptides were identified by using rabbit antiserum prepared against purified mouse caseins (15) and the immunoblot procedure for gel-fractionated proteins (17, 18).

Detection of Keratin by Indirect Immunofluorescence. COMMA-1D cells were grown subconfluently on glass coverslips in 35-mm (diameter) Petri dishes. The cells were fixed and stained for the detection of keratin by indirect immunofluorescence as described (19) and were examined on a Leitz epi-fluorescence microscope. Rabbit and guinea pig antisera to mouse keratins were generously provided by B. B. Asch. The preparation and specificity of the antisera used have been described elsewhere (19).

Transplantation of COMMA-1D Cells into Syngeneic Hosts. COMMA-1D cells were removed from one 100-mm (diameter) Petri dish by enzymatic dissociation with trypsin/EDTA and collected by centrifugation in the presence of DME medium with 10% fetal bovine serum and insulin (5 μg/ml). The resulting pellet was resuspended carefully in 0.25 ml of serum-free medium consisting of DME medium with the growth supplement. Aliquots (10 μl) representing 2.5–3.0 × 10^5 cells were injected into cleared mammary fat pads of 3-wk-old syngeneic BALB/c female mice. After 4–8 wk, the mammary fat pads were removed from the virgin mice and processed as whole mounts as described by Medina (20).

RESULTS

Growth and Morphology of COMMA-1D Cells *in Vitro*. COMMA-1D cells displayed a typical cuboidal epithelial-like morphology in culture and formed monolayers with the cells in close contact with each other (Fig. 1). The cells grew to a saturation density of 2 × 10^5 cells per cm^2. Many blister-like structures or "domes" appeared in confluent cultures (Fig. 1, arrows). Dome formation is believed to result from fluid secretion by mammary epithelial cells cultured on a plastic substrate. COMMA-1D cells failed to grow in suspension culture under conditions that foster the formation of multicellular spheroids by mammary tumor cells. The cells retained a near-diploid karyotype of 42 or 43 chromosomes per nucleus. COMMA-1D cells have not been cloned. COMMA-1D cells grown in antibiotic-free medium for 2 wk were free of mycoplasma contamination, as determined by staining with bisbenzimide (Hoechst 33258 stain) (21).

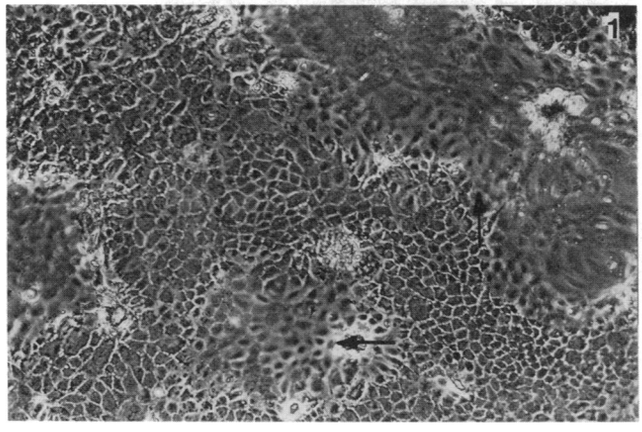

FIG. 1. Monolayer of COMMA-1D cells at saturation density. Several domes (arrows) are present. (Phase contrast; ×77.)

When embedded in collagen gels, COMMA-1D cells produced predominantly three-dimensional duct-like outgrowths (Fig. 2*A*), similar to those produced by normal mouse mammary cells (Fig. 2*B*). In addition, a small percentage (<5%) of outgrowths was characterized by a network of thin, irregularly packed branches (data not shown). This minor morphological outgrowth pattern was seen also with normal mammary cells and the significance of this variant pattern is not known.

COMMA-1D cells contained typical keratin intermediate filaments by immunofluorescence (Fig. 3*A*). Positive staining for keratin was obtained with the rabbit antiserum that reportedly reacts with both ductal epithelial and myoepithelial cells, whereas the guinea pig antiserum that is specific for keratin in myoepithelial cells failed to react (Fig. 3*B*).

Synthesis of Casein Polypeptides *in Vitro*. COMMA-1D cells at passages 3 and 7 were grown in collagen, exposed to induction medium as described in *Materials and Methods*, and then examined for the expression of specific casein proteins. Casein was inducible in COMMA-1D cells (Fig. 4, lanes D and E) and the casein polypeptides produced were indistinguishable from those of normal mouse mammary cells derived from virgin mice (Fig. 4, lane C). The four main types of mouse caseins that were purified from mouse milk are shown in lane B. The most abundant intracellular casein detected in both normal mammary cells and COMMA-1D

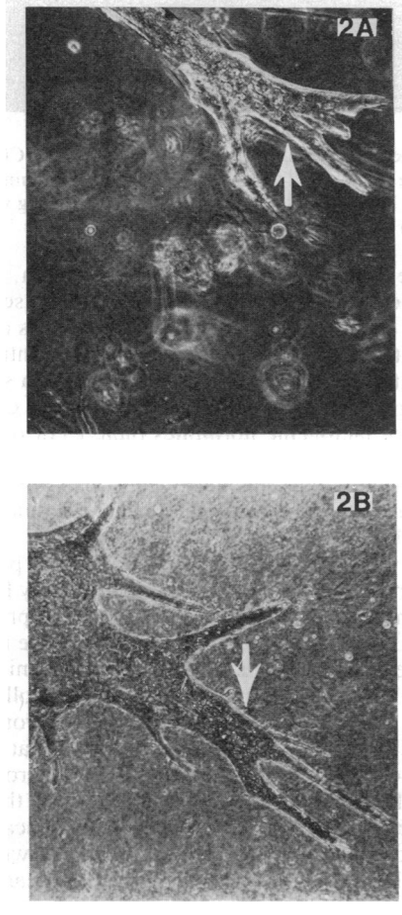

FIG. 2. (*A*) Outgrowth of COMMA-1D cells (arrow) embedded in a collagen gel. (Phase contrast; ×77.) (*B*) Outgrowth of normal mouse mammary cells (arrow) taken from a primary culture and embedded in a collagen gel. (Phase contrast; ×77.) Note the similar duct-like morphology of both the outgrowths.

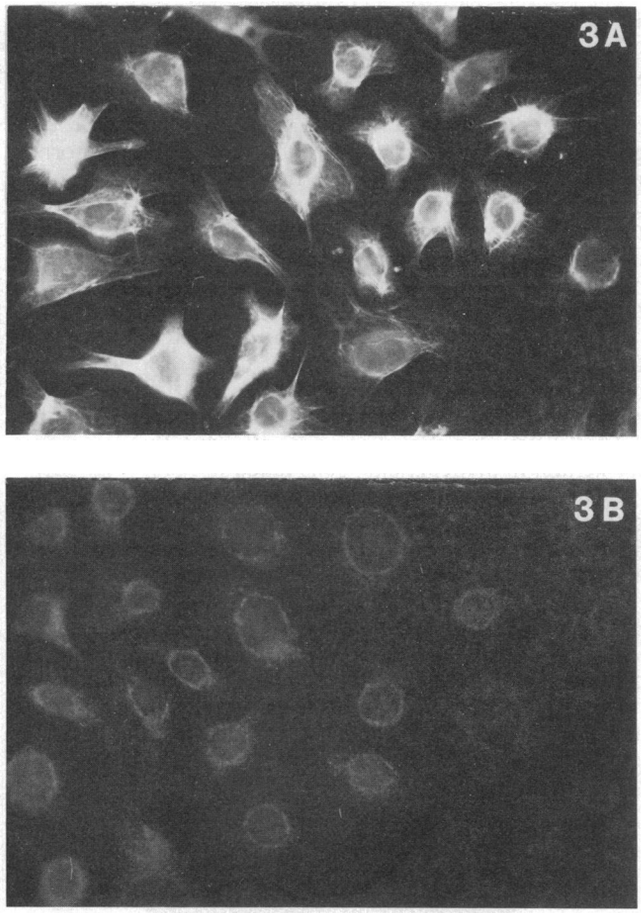

FIG. 3. Indirect immunofluorescent staining of COMMA-1D cells in culture by antisera to keratin. (*A*) Positive staining with rabbit antikeratin antiserum. (×240.) (*B*) Negative staining with guinea pig antikeratin antiserum. (×240.)

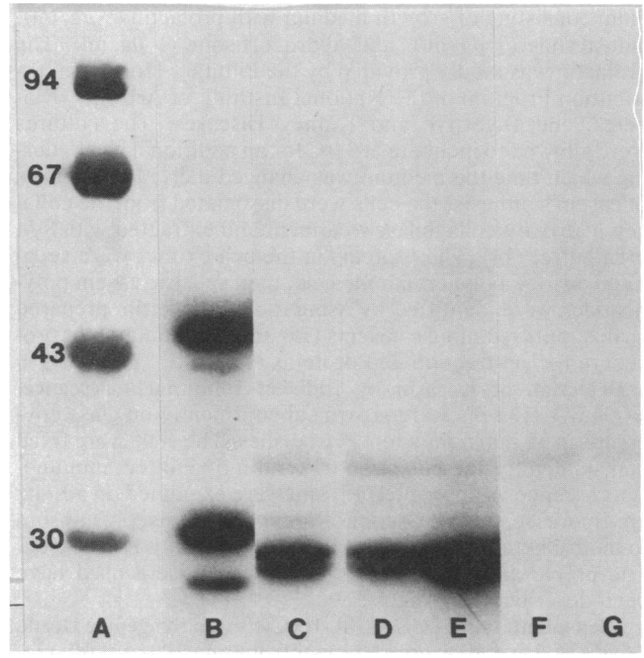

FIG. 4. Immunoblot analysis of caseins synthesized by normal mouse mammary cells and COMMA-1D cells embedded in collagen. Lane A, molecular weight standards, with values expressed as $M_r \times 10^{-3}$. Lane B, casein polypeptides detected by rabbit antiserum to mouse milk caseins. Lane C, normal mouse mammary cells from virgin BALB/c mice; cells were embedded in collagen and, after 3 wk, treated with induction medium for 9 days. Lane D, COMMA-1D cells, treated as in lane C. Lane E, COMMA-1D cells, same as lane C, except treated with induction medium for 16 days. Lane F, COMMA-1D cells, same as lane C, except no treatment with induction medium. Lane G, COMMA-1D cells grown on plastic for 3 wk and treated with induction medium for 9 days.

cells was the M_r 29,000 polypeptide. In addition, two other casein polypeptides were also present. The antiserum used in this study does not react with cellular proteins other than casein or with mouse mammary tumor virus antigens (unpublished data). The COMMA-1D cells failed to synthesize detectable amounts of casein when cultured in collagen in the absence of lactogenic hormones (lane F) or on a plastic substrate in the presence of lactogenic hormones (Fig. 4, lane G). Normal mouse mammary cells also fail to synthesize detectable amounts of casein under these culture conditions (unpublished data).

Growth of COMMA-1D Cells *in Vitro*. Cells at passages 4, 7, and 11 were injected into the cleared mammary fat pads of 3-wk-old syngeneic BALB/c female mice. Approximately 2.5–3.0 × 10^5 cells were injected per fat pad. The mice from the first experiment were maintained as virgin animals for 8 wk, at which time the mammary fat pads were collected and processed as whole mounts. All 12 transplants from passage 4 grew in recipient mice. The mean percentage fat pad filled was 88% ± 4.5%. The morphology of the outgrowths was predominantly ductal throughout the extent of the fat pad (Fig. 5). Occasional small alveolar buds were scattered on the ducts and end buds were evident on the growing tips of outgrowths that had not completely filled the mammary fat pad (Fig. 5). Eight and 6 transplants were examined 4 wk after transplantation derived from passages 7 and 11, respectively. Thirteen of 14 transplants were successful and produced ductal outgrowths. The results of these experiments are summarized in Table 1. In a preliminary experiment, 6 of 6 transplants also responded to the physiological stimulus of pregnancy with the development of lobuloalveolar differentiation in 16-day pregnant mice.

DISCUSSION

This report documents the phenotypic characteristics of a mouse mammary cell line, COMMA-1D, that retains morphogenic and functional properties of normalcy even after multiple subcultures *in vitro*. COMMA-1D cells retain an epithelial morphology and continue to form domes *in vitro* after 15 subcultures. The cells are also inducible for casein synthesis, contain keratin intermediate filaments and, most

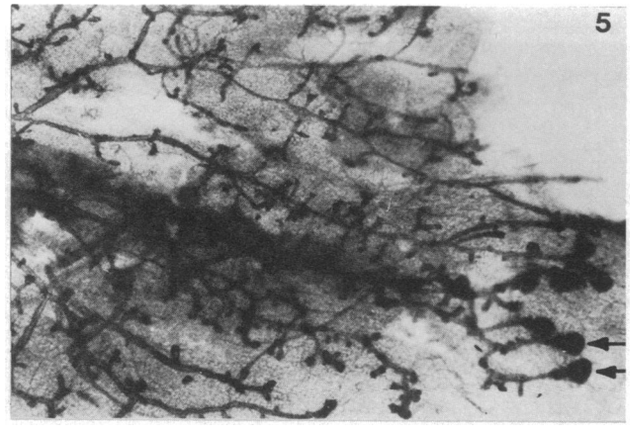

FIG. 5. Ductal growth of COMMA-1D cells in the mammary fat pad 8 wk after transplantation. Note end buds at end of ducts (arrows). (× 9.5.)

Table 1. Summary of the phenotypic properties of mammary epithelial cell line COMMA-1D

Property	Cell passage(s) tested	Result
In vitro		
Saturation density on plastic	7	2×10^5 cells per cm^2
Growth in suspension culture	7, 9	Negative
Modal chromosome number	3, 14	42 or 43
Presence of keratin filaments	7	Positive*
Synthesis of casein		
On plastic	3, 7	Negative
Within collagen	3, 7	Positive
In vivo		
Growth in mammary fat pads	4, 7, 11	Ductal outgrowths†

*Keratin detected by using antiserum specific for mammary epithelial cells; no reaction was obtained with a keratin antiserum specific for myoepithelial cells.
†25/26 fat pads injected.

importantly, form ducts upon injection into their normal environment—i.e., the mammary fat pad. Furthermore, preliminary observations demonstrate that the cells contain desmosomes by transmission electron microscopy (data not shown) and synthesize a mouse mammary tumor virus-specific antigen with an estimated M_r of 67,000 (unpublished observations). These characteristics, as a whole, provide unequivocal criteria for the identification of a mammary epithelial cell population that exhibits significant normal properties (Table 1).

The positive staining of the COMMA-1D cells with rabbit anti-keratin antiserum and the negative staining with guinea pig anti-keratin antiserum suggest that few, if any, myoepithelial-like cells are part of the COMMA-1D cell population (the latter antiserum identifies myoepithelial cells specifically). Since COMMA-1D cells will produce intact ductal structures after transplantation *in vivo*, these cells may represent progenitor cells in the mammary cell lineage and may be useful for studies of mammary gland differentiation.

Other murine cell lines that are considered to represent presumptive "normal" mammary cells lack one or more significant phenotypic properties of mammary epithelia. The C57MG line established by Vaidya *et al.* (7) is nontumorigenic in nude mice and can be transformed by mouse mammary tumor virus and 7,12-dimethylbenz[*a*]anthracene treatment in combination (8). However, its morphogenic potential in the mammary fat pad and its differentiation potential *in vivo* or *in vitro* have not been examined. Limited data available suggest that C57MG cells do not grow *in vivo* (refs. 7 and 8; unpublished data). Another example is the CL-S1 line established from preneoplastic mammary tissue (9). It is epithelial *in vitro*, forms domes in high density culture of low passaged cells, contains keratin intermediate filaments by indirect immunofluorescence, and is marginally inducible for casein (unpublished data). However, it does not produce any type of outgrowth upon transplantation into the cleared fat pads of syngeneic mice. A third cell line is the NMuMG mammary line, established from an adult NAMRU female mouse (22). The cells are epithelial *in vitro* and characterized by dome formation, desmosomes, and tonofilaments; however, it produced benign cystadenomas at passages 6 and 17 when injected into syngeneic mice (22).

Attempts to study mammary cells in culture have been fraught with disappointments and frustrations. Mammary cells from either mice or humans generally can be carried through a maximum of four subpassages before their *in vitro* proliferative capabilities stagnate (23, 24). In the majority of cases, the *in vivo* proliferative, morphogenic, and functional potentials were not examined, and, thus, the normalcy of the presumptive mammary epithelial cells was not established. Mammary cells successfully cultured for >4 passages *in vitro*, however, were either tumorigenic (3–6), unable to produce any glandular outgrowth *in vivo* (9, 22), or uncharacterized for *in vivo* properties (7, 8). Recently, a cell culture system that might prove to yield normal mammary epithelial cells after prolonged passage was reported by Hammond and co-workers.‡ They have been successful in culturing normal human mammary cells for up to 16 passages *in vitro*. As yet, there is no documentation of the degree of differentiation of these cells. Thus, up to the present time, the propagation of mammary epithelial cells in monolayer culture has not been sufficiently successful for the study of normal or neoplastic developmental events.

At this time, it is unclear why the COMMA-1D cell line is fluorishing. The initial culture methodology was not unique, although in contrast to previous studies, this cell line has been maintained primarily in a low concentration of serum (1%) and DME medium supplemented with a variety of growth factors. Whether this growth medium formulation is a significant factor or purely coincidental in allowing the establishment of this cell line is not known. It is interesting that the growth rate of the mammary cells rapidly increased following the intermittent treatment of the cell monolayer with EDTA/trypsin to eliminate fibroblasts and the switch from high to low levels of serum in the medium. However, we have not been successful in establishing additional cell lines using this culture methodology from three subsequent attempts over the past year.

The availability of this established mammary epithelial cell line should afford the opportunity to investigate mammary gland biology and differentiation *in vitro*. The cells should also provide the appropriate target tissue to examine *in vitro* the events of transformation produced by chemical and viral agents.

‡Hammond, S. L., Ham, R. G. & Stampfer, M. R., *Proceedings of the International Association for Breast Cancer Research*, March 20–24, 1983, Denver, CO, abstr. p. 59.

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1. Medina, D. (1978) in *Breast Cancer*, ed. McGuire, W. L. (Plenum, New York), Vol. 2, pp. 47–102.
2. Medina, D. (1982) in *The Mouse in Biomedical Research*, eds. Foster, H. L., Small, J. D. & Fox, J. G. (Academic, New York), Vol. 4, pp. 373–396.
3. Yagi, M. J. (1973) *J. Natl. Cancer Inst.* **51**, 1849–1860.
4. Dexter, D. L., Kowalski, H. M., Blazar, B. A., Fligiel, Z., Vogel, R. & Heppner, G. H. (1978) *Cancer Res.* **38**, 3174–3181.
5. Danielson, K. G., Anderson, L. W. & Hosick, H. L. (1980) *Cancer Res.* **40**, 1812–1819.
6. Butel, J. S., Dudley, J. P. & Noonan, C. A. (1980) in *Cell Biology of Breast Cancer*, eds. McGrath, C. M., Brennan, M. J. & Rich, M. A. (Academic, New York), pp. 317–345.
7. Vaidya, A. B., Lasfargues, E. Y., Sheffield, J. B. & Couthino, W. G. (1978) *Virology* **90**, 12–22.
8. Howard, D. K., Schlom, J. & Fisher, P. B. (1983) *In Vitro* **19**, 58–66.
9. Anderson, L. W., Danielson, K. G. & Hosick, H. L. (1979) *In Vitro* **15**, 841–843.

10. Asch, B. B. & Medina, D. (1978) *J. Natl. Cancer Inst.* **61,** 1423–1430.
11. Medina, D. & Oborn, C. J. (1980) *Cancer Res.* **40,** 3982–3987.
12. Moorhead, P. S. (1973) in *Tissue Culture Methods and Applications*, eds. Kruse, P. F., Jr., & Patterson, M. K., Jr. (Academic, New York), pp. 768–773.
13. Seman, G. (1980) *J. Tissue Culture Methods* **6,** 141–143.
14. Tonelli, Q. J. & Sorof, S. (1981) *Differentiation* **20,** 253–259.
15. Durban, E. M., Medina, D. & Butel, J. S. (1982) *J. Cell Biol.* **95,** 51a (abstr.).
16. Yang, J., Richards, J., Bowman, P., Guzman, R., Enami, J., McCormick, K., Hamamoto, S., Pitelka, D. & Nandi, S. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 3401–3405.
17. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.
18. Burnette, W. N. (1981) *Anal. Biochem.* **112,** 195–203.
19. Asch, B. B., Burstein, N. A., Vidrich, A. & Sun, T. T. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 5643–5647.
20. Medina, D. (1973) *Methods Cancer Res.* **7,** 3–53.
21. Chen, T. R. (1977) *Exp. Cell Res.* **104,** 255–262.
22. Owens, R. B., Smith, H. S. & Hackett, A. J. (1974) *J. Natl. Cancer Inst.* **53,** 261–269.
23. White, M. T., Hu, A. S. L., Hamamoto, S. T. & Nandi, S. (1978) *In Vitro* **14,** 271–281.
24. Stampfer, M., Hallowes, R. C. & Hackett, A. J. (1980) *In Vitro* **16,** 415–425.