To Grow Mouse Mammary Epithelial Cells in Culture

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ABSTRACT   Normal mouse mammary epithelial cells from Balb/c mice were successfully cultivated on tissue culture plastic with lethally irradiated LA7 feeder cells. The feeder cells also promoted colony formation from single mouse mammary cells, and the fraction of cells that formed colonies was proportional to the density of feeder cells. The mouse mammary cells could be passaged at least 8–12 times as long as new feeder cells were added at each passage. The cells now in culture have doubled in number at least 30 times, but the in vitro lifespan is not yet known. The cultures of mouse cells maintained by this technique never became overgrown with fibroblasts and numerous domes formed in the cultures.

Epithelial cells have long resisted growing in culture, but within the last few years several advances have been made in the culturing of several types of epithelial cells from normal as well as neoplastic tissue. By supplementing media with known hormones and growth factors instead of serum, epithelial proliferation has been promoted at the same time that fibroblast overgrowth of the cultures has been inhibited (1, 2). By culturing epithelial cells on collagen or extracellular matrices growth as well as performance of differentiated functions has been promoted (3–10). In addition, feeder cells, inactivated from proliferation by radiation or other means, have also aided in promoting growth of some epithelial cells either by providing a more normal substrate for the epithelia or by conditioning the medium (11–16). Various combinations of these techniques have been used with varying success for different types of epithelial cells. The culture system developed by Rheinwald and Green (13) for human keratinocytes probably has succeeded in promoting the longest proliferative lifespan of epithelial cells in culture. With irradiated 3T3 cells as a substratum and medium supplements of epidermal growth factor, hydrocortisone, and fetal calf serum the cells can divide for more than 50 doublings in cell number or 150 generations. This success is equaled by a more recently developed technique for mesothelial cells in which the irradiated 3T3 feeder cells could be omitted (17). More typically, however, only 4–6 passages with 4–5 doublings per passage has been the limit for other epithelia including rabbit tracheal (18), human bronchial (11), and human mammary (19–22).

The culture system we describe here for mouse mammary epithelial cells from normal mammary gland differs from most of the other systems in that the feeder layer of cells is epithelial rather than fibroblastic in origin (12, 20, 22, 23).

Although the growth medium is yet relatively undefined the mouse mammary cells that we have in culture have so far lived through at least 12 passages or 30 doublings in cell number or more than 85 cell generations. A preliminary report of these data have been orally reported (24).

MATERIALS AND METHODS

Growth Medium: Cells were grown in Dulbecco's modified Eagle's medium (DME) (KC Biologocal, Lenexa, KS) with a 4.5 g/liter glucose, 110 mg/liter sodium pyruvate, supplemented with 10 µg/ml insulin, 0.2 mM (120 U/ml) penicillin G, and 0.05 mM streptomycin sulfate (Sigma Chemical Co., St. Louis, MO), 20 mM HEPES buffer, 1.1 g/liter NaHCO$_3$, and 10% fetal bovine serum (Sterile Systems, Logan, UT).

Primary Cells: The mammary glands were removed from 6–8 wk-old female Balb/c mice under sterile conditions and chopped into very fine bits with a razor blade. These bits were placed into 0.1% collagenase (Grade III, Worthington Biochemical Corp., Freehold, NJ) in DME for overnight digestion at 37°C. The next morning the digestion mixture was drawn through a 10-ml pipette numerous times to further break the clumps. The cells and clumps were then spun into a pellet by centrifuging for 7 min at 100 g. The pellet was washed twice with PBS and spun down. The pellet was resuspended in PBS and filtered through a 95-µm Nitex filter to eliminate many of the fibroblasts which as single cells fell through the filter. The epithelial clumps that had settled on the top of the filter were washed off, spun into a pellet, resuspended in growth medium with 15% DMSO and frozen until use. After defrosting, the cells were placed in plastic flasks where the clumps adhered to the bottom after 1–2 d and the clumps slowly spread out onto the plastic forming a single cell sheet.

Feeder Cells: Rat mammary tumor cells of the LA7 line were generously given to us for this use by Dr. Renato Dulbecco, The Salk Institute (San Diego, CA). The LA7 cells are a clonal derivative of the Rama 25 line which came from a Sprague-Dawley rat treated with dimethylbenz(a)anthracene (25, 26). Before combining with normal mouse mammary cells in culture, the LA7 cells were lethally irradiated by [137]Cs. The concentration of feeder and normal mouse cells together was 3–5 × 10^5 cells/cm^2 unless otherwise indicated.

Cloning and Colony-forming Efficiency: Normal mouse mam-

mary cells residing on plastic were dislodged by a few minutes incubation in a trypsin-EDTA PBS solution. After dilution of these cells into a few milliliters of growth medium, the cells were drawn into and out of a 10-ml pipette 5–10 times to disperse clumps. Further dissociation into single cells was achieved by drawing the cell solution twice through a 23 gauge needle. A further dilution of the cell suspension was counted both on a hemocytometer and by a Coulter ZBI particle counter (Coulter Electronics, Hialeah, FL). The cells were serially diluted and mixed with the appropriate number of feeder cells such that ~1,000 mouse mammary cells were plated into each T-75 flask for colony-forming experiments or an average of ½ cell per well in 96-well plates (Costar, Cambridge, MA). Both types of cultures were fed with fresh growth medium three times per week.

Cell Characterization: The presence of keratin in the tissue cultures was ascertained with a rabbit antiserum to human epidermal keratin. Colonies of putative mouse mammary epithelial cells in passage 2 as well as colonies of fibroblasts in passage 18 that had come from overgrowth of primary mammary tissue plated on plastic without feeders were exposed to antiserum and stained by the peroxidase-antiperoxidase technique. The keratin antiserum was generously provided to us by Dr. Tung-Tien Sun, Johns Hopkins Medical School (Baltimore, MD).

The species from which the tissue culture cells originated was determined by an antibody to species-specific antigens (rats and mouse, in this case) on the cell surface membrane. Briefly, the antisera were obtained from rabbits that had been immunized with the cells from the species of interest and the antisera were conjugated with fluorescein isothyionate (27). The coupled antibody was mixed with a suspension of the tissue culture cells of interest and viewed by ultraviolet light microscopy to determine the fraction of each type of cell present.

Isoenzyme analysis of the tissue culture cells was also used to identify the species of origin. This technique, described elsewhere (28), depends upon the fact that isoenzymes from different species migrate at different rates with agarose electrophoresis. The enzymes measured here were lactate dehydrogenase, glucose-6-phosphate dehydrogenase, nucleoside phosphorylase, and malate dehydrogenase. Briefly, living cells were disrupted by freeze thawing a few times in ice and methanol, the cell membranes were removed from the preparation by centrifugation, small amounts of the cytosol were placed on agarose electrophoresis film, and after electrical separation the films were stained and distance of migration was analyzed (28).

Photography: Photographs of the cultured cells were taken with a Polaroid back attached to an inverted Nikon phase microscope and with Polaroid black and white Land film, Type 084. When a certain field was to be photographed on successive days, the field was marked with a diamond needle (Carl Zeiss, Inc., Thornwood, NY) attached to the nosepiece of the microscope. To prepare cultures for electron micrography the mouse mammary cells were plated with irradiated LA7 feeder cells into 35-mm petri dishes at a ratio of 1:3, respectively so that the mixture formed confluent monolayers on the dishes. The cells were fed three times a week during the next 2-mo-incubation period, a time long enough to ensure that almost all the irradiated feeder cells had died, leaving a monolayer of mouse mammary cells. The dishes were washed twice with PBS and fixed with buffered glutaraldehyde and embedded for electron microscopy as described previously (29).

RESULTS

Primary mouse mammary colonies, when plated on plastic, attached within 2 or 3 d as clumps from which the cells spread out onto the plastic as round single cell sheets. During the spreading some cells divide, but division ceased after the colony had spread out. When such colonies were incubated for long periods, the cells slowly detached from each other and moved apart. Upon even longer incubation the cells disappeared: they detached from the plastic and probably died (Table I). If, however, lethally-irradiated LA7 feeder cells were added to the culture of colonies, the mouse mammary cells within these colonies proliferated vigorously to form very large colonies (Table I). The stimulation of the cell growth occurred whether the feeder cells were added in the early or late stages of the colony spreading. A confluent monolayer of the mouse mammary primary cells could be obtained if enough colonies were plated and if the appropriate number of feeder cells was added. These cultures could be passaged with success as long as new feeder cells were added to each new culture (Fig. 1). At present we have cells in culture in the

12th passage that are still able to proliferate.

Not only did primary mouse cell cultures cease proliferation without feeders but mouse cells that had been carried for several passages with feeders and then were plated without feeders would not proliferate (Figs. 2 and 3). We had carried one group of cells for eight passages, including one cloning with feeders, and then we plated the cells from each of the 14 obtained clones into flasks without feeders at about ¼ of their maximum density. After 3 wk only one of these clones seemed to have proliferated; the other 13 were at about the same density as plated. At this time random areas on eight flask bottoms (i.e., eight different clones) were marked with three circles each so that the identical spots could be studied photographically with time. Feeder cells were then added to four of these flasks. After about five more weeks, a time when almost all feeder cells had died, the mouse cells to which the feeders had been added had multiplied by about a factor of four (Figs. 2 and 3). Of the four clones that received no feeder cells, three proliferated very little or not at all (Fig. 3). Feeder cells were subsequently added to two of these cultures, where-

TABLE I

Growth of Primary Mouse Mammary Colonies with Feeders*

Treatment	Colony	No. of cells in colony day 8	No. of cells in colony day 30	Cell multiplication	Cell doublings
Flask 1[*]	1	122	0	none	none
	2	62	3	none	none
	3	205	5	none	none
	4	89	1	none	none
Flask 2[§]	1	146	8,715	59.7×	5.9
	2	157	3,564	22.7×	4.5
	3	972	9,424	9.7×	3.3

* Primary mouse mammary cells were plated into two flasks on day 0. On day 8 the position of a few colonies was marked on the flasks and the colonies were photographed. Lethally irradiated LA7 feeder cells were then added to one flask. On day 30 the same colonies of mouse cells were again photographed. The number of cells in each colony was counted on the photographs.
[*] No LA7 feeder cells added.
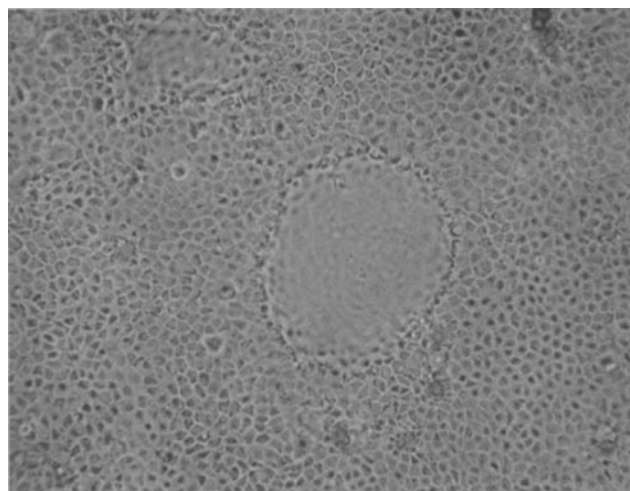[§] LA7 feeder cells added on day 8.

FIGURE 1 Mouse mammary epithelial cells in passage 5. The cells were plated along with irradiated LA7 feeder cells so that the resulting mixed monolayer consisted of ~7% mouse mammary cells. After 6 wk almost all of the irradiated LA7 cells had died and the monolayer (shown here) consisted almost completely of mouse mammary cells that formed numerous domes. × 100.

<div align="center">

**feeder cells
added**   **no feeder cells
added**
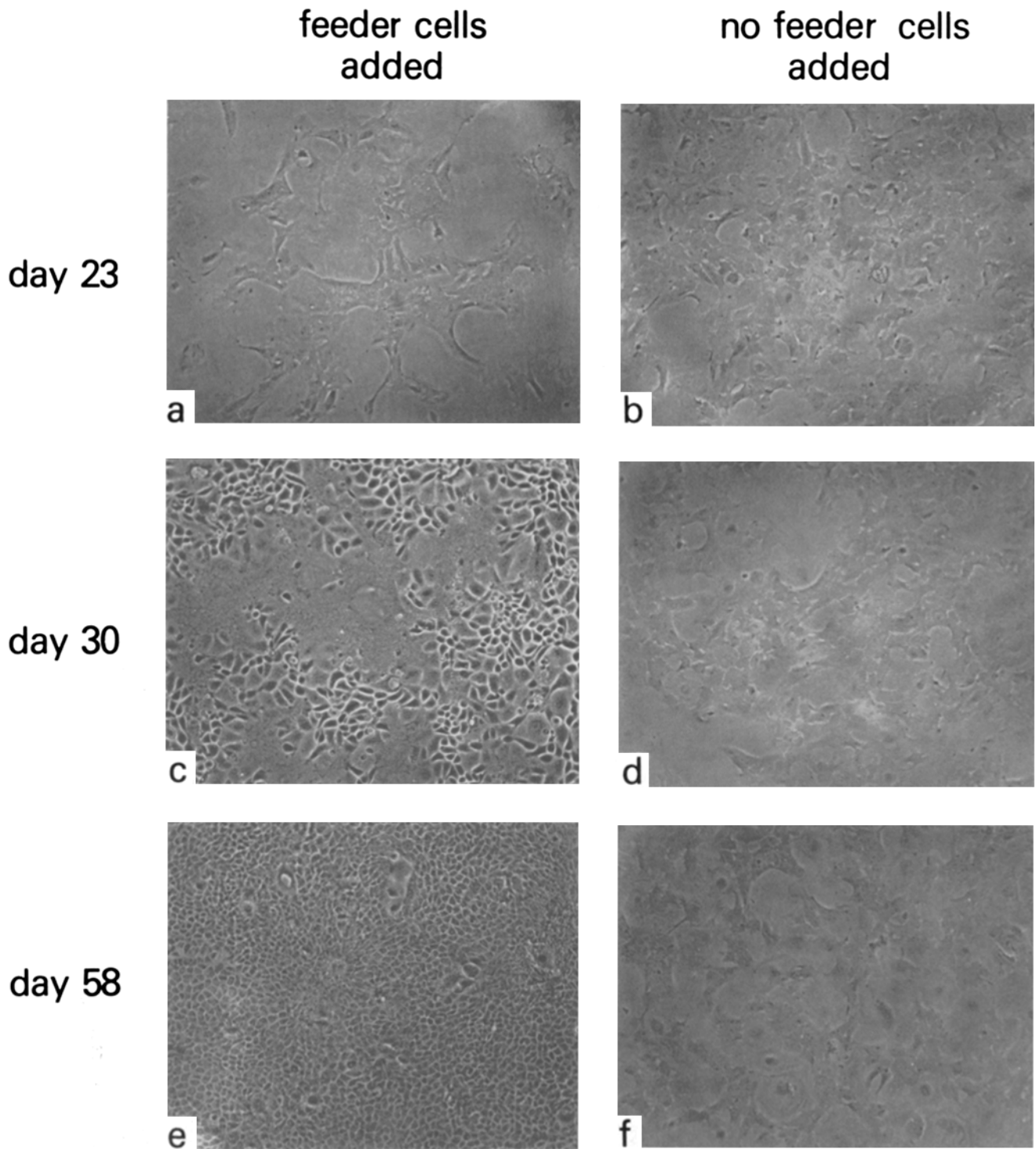
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day 23

day 30

day 58

FIGURE 2   Two mouse mammary clones after seven passages with LA7 feeder cells. Each clone was plated at one-fourth maximum density in passage 8. On day 23 after plating, several areas of each flask were marked and photographed. Then irradiated LA7 feeder cells were added to one flask. (a, c, and e) The same photographic field of a clone on days 23, 30, and 58 respectively after plating. Lethally irradiated LA7 feeder cells were added to this culture on day 23 after the photograph was taken. The many large cells in c and the few large cells in e are those feeders that had not yet died. (b, d, and f) The same photographic field of another clone on days 23, 30, and 58 respectively after plating. No feeder cells were added to this flask. × 76.

upon they proliferated to maximum density (Fig. 3). The fourth clone that received no feeders was nevertheless able to increase its numbers by about a factor of three after 5 wk. Subsequently, proliferation within this clone resulted in a cell number that represented the maximum cell density for these mouse mammary cells in culture (Fig. 3). The fact that one

clone was able to proliferate without feeder cells might suggest that after a few passages the normal mouse cells may become independent of feeders. This may be the case, but we suspect, rather, that this growth phenotype may have been inadvertantly selected for in this population of cells because these particular cells were originally maintained in culture without

feeders for several months before they were cloned and passaged with feeders. Some of our newer cultures that have always been maintained with feeder cells as far as passage 5 have not shown any sign that they can proliferate in culture
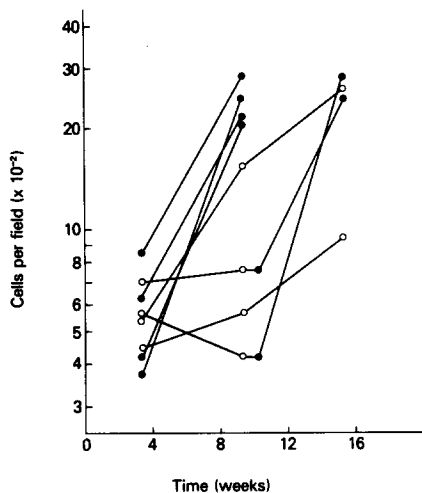

FIGURE 3 Proliferation of cells in eight mouse mammary clones with or without LA7 feeder cells. Photographs of the same fields were taken at successive times after eight mouse mammary clones had been plated at one-fourth maximum density as in Fig. 2. The number of cells in each photograph was counted and plotted here. O, no feeder cells added; ●, feeder cells added.

without feeders (data not shown).

This system of growing normal mouse mammary cells with irradiated LA7 feeders cells was also useful as a colony-forming assay at any passage after the primary culture. The cells were dispersed into an essentially single cell suspension with trypsin + EDTA in PBS and when a small number of these mouse cells was plated with feeders cells, visible colonies formed after 3 wk (Fig. 4). The percentage of cells that formed colonies depended upon the density of the feeder cells with which they were plated (Fig. 5). The higher the feeder density, the more mouse mammary cells proliferated to form colonies. The highest colony forming efficiency was ~14%, a number that has been obtained with fair reproducibility by us in several other experiments where the feeder density was above 60% maximum. The colonies that formed with this single cell plating technique had to arise from the mouse mammary cells rather than from the LA7 feeder cells because colonies never formed in control flasks plated with LA7 feeder cells alone.

Fibroblast cells from the dissociated mammary gland never overgrew the epithelial cell cultures when the latter were plated with enough LA7 feeder cells. Not many fibroblasts grew in the primary cultures, and by passage 3 fibroblasts were difficult to find. The number of fibroblasts that proliferated was inversely proportional to the density of the LA7 feeders plated with the culture, but when they did grow they were easily identified not only by cell morphology at the light microscope level (Fig. 4, b and c) but by the shape of the colonies formed (Fig. 4a). The epithelial colonies were round with distinct
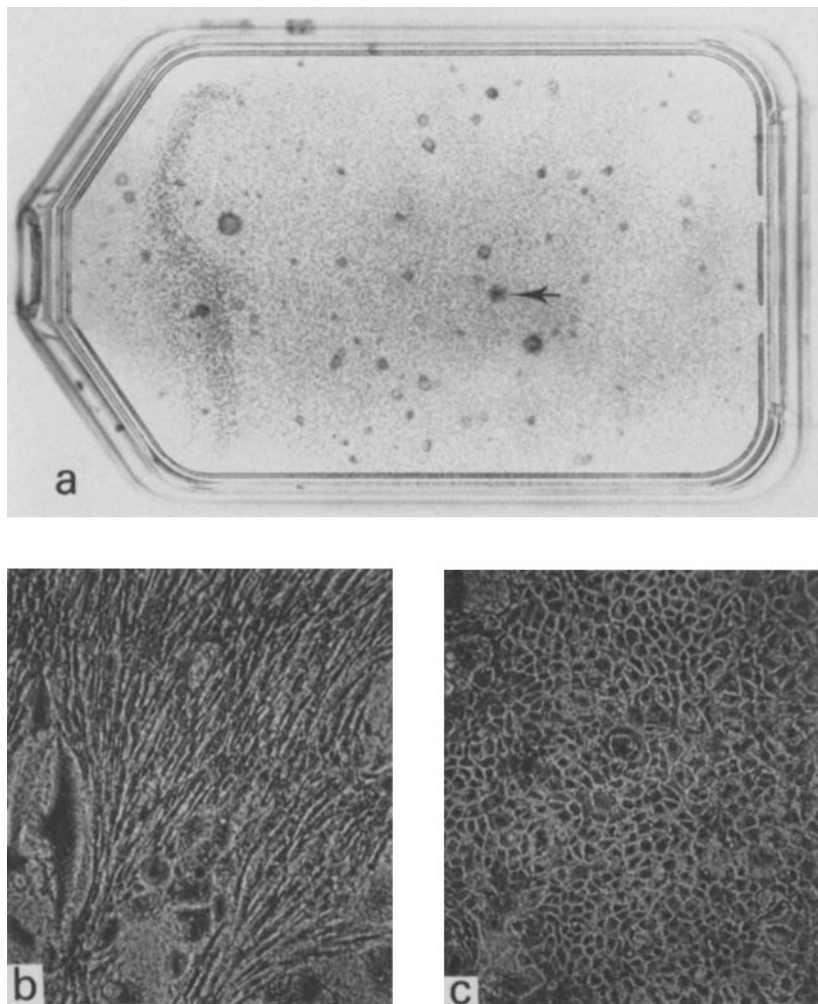

FIGURE 4 Colony forming assay for normal mouse mammary cells. (a) A T75 flask fixed and stained after 7 wk of incubation. 31 epithelial colonies and one fibroblast colony (arrow) developed from single cells plated within a bed of irradiated LA7 feeder cells. (b) Edge of the fixed fibroblast colony in a. (c) A portion of a fixed epithelial colony in a. (A) × 0.80; (b and c) × 74.

borders whereas the fibroblast colonies had various shapes and indistinct borders.

The cells that have grown with this feeder system were determined to be epithelial by several criteria. The epithelial morphology of the cells by light microscopy was confirmed by electron microscopy which revealed apical microvilli and tight intercellular junctions (Fig. 6). Another indication that the cells were epithelial was the formation of numerous domes within the colonies or monolayers formed by the cells indicating that the cells transported solutes across the cell sheet. We also determined that the putative mouse mammary cells contained keratin, which has been found in all epithelial cells (30, 31). For this procedure several colonies from a flask such

MOUSE MAMMARY CELL COLONIES
WITH LA7 FEEDERS (6000 rads)
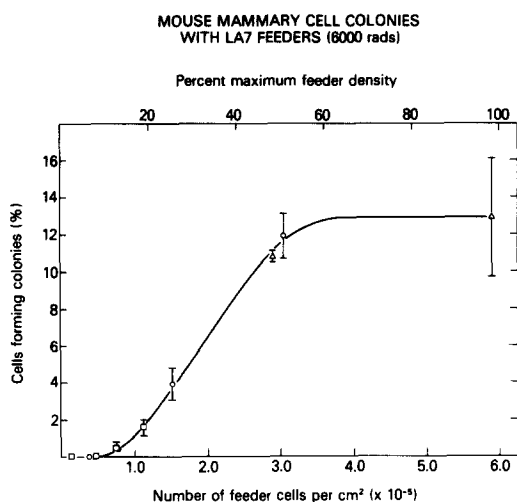
Percent maximum feeder density

FIGURE 5   The relationship of colony formation of single mouse mammary cells to the density of irradiated feeder cells. Mouse mammary cells in a single cell suspension were plated along with lethally irradiated LA7 feeder cells at different densities. The cultures were fixed, stained, and colonies counted 3 wk after plating. Each symbol type represents results from one experiment.

as that of Fig. 4 as well as fibroblast colonies originally from mouse mammary gland and now in passage 18 were exposed to antikeratin antibodies. Only the putative mouse mammary epithelial colonies reacted with the antibodies to give a definite brown orange color characteristic of the immunoperoxidase stain. The fibroblast colonies, on the other hand, contained no hint of this color. In vivo the mammary gland consists of ductal, alveolar, and myolepithelial cell types, but according to the above criteria, including morphology, all cells in these cultures were similar. Ductal and alveolar cells far outnumber myoepithelial cells in the mammary gland, and if a minor population of myoepithelial cells was present in our cultures, we were not able to detect it.

These cells were proven to be mouse cells rather than rat mammary cells from the feeder layer by three means. First, when the mouse mammary cells were seeded sparsely enough to form colonies such as those in Fig. 4, a few flasks of feeder cells alone were always prepared alongside as controls. Colonies never formed in these control flasks, indicating that the colonies in the flasks of interest did not arise from feeder cells that had survived the radiation but rather from the mouse cells. In addition, when the putative mouse mammary cells in passage 7 were reacted with a flourescently labelled antibody that reacts only with mouse cells (27) 95% of the cells in the culture fluoresced. In the same culture 5% of the cells reacted with the rat antiserum. These 5% of cells that fluoresced with the anti-rat antibody were irradiated cells from the LA7 feeder layer that had not yet died. Taking into account the number of mouse mammary cells seeded into the flask with the feeder cells at the beginning of the cultures, the number of fluorescent cells, and total cell number at the time of the assay, we could calculate that the number of mouse cells in that culture increased by a factor of about 12 or about 3.5 doublings in cell number. These putative mouse cells in passage 7 were also proven to be mouse by electrophoretic analysis of four enzymes. The rates of migration of these enzymes differ from species to species, and can be used to
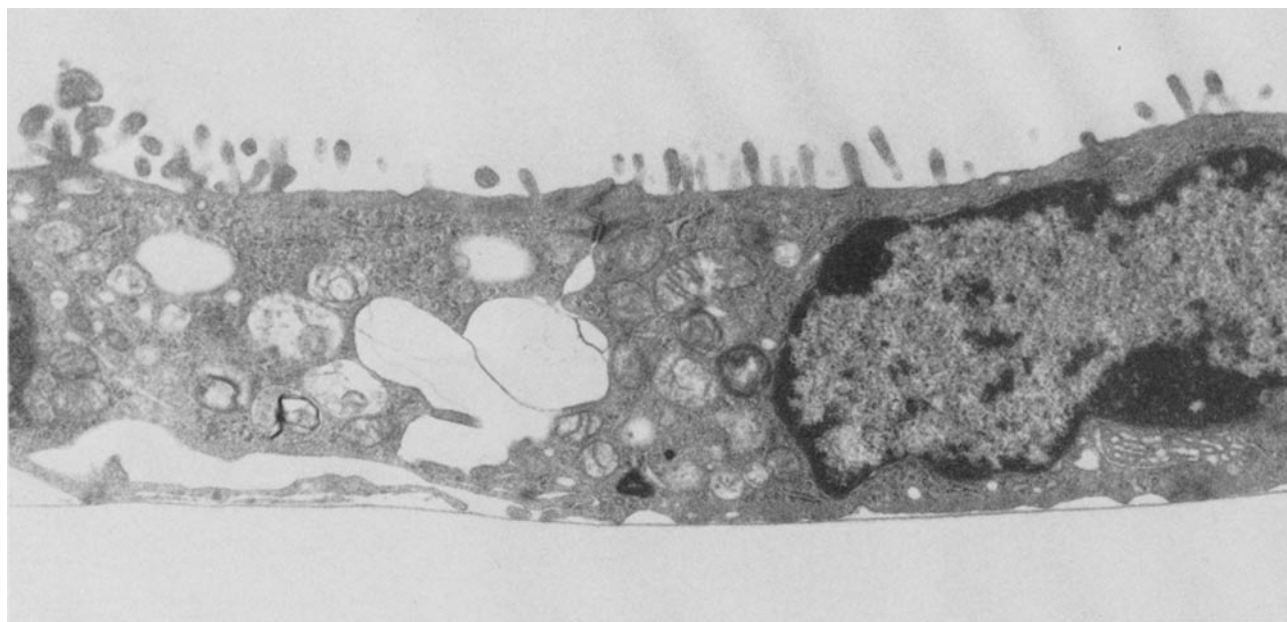

FIGURE 6   Cross section of normal mouse mammary cells by electron microscopy. These cells in passage 6 after more than 15 doublings in culture have microvilli on their apical surfaces and join each other by tight occluding junctions and desmosomes × 11,700.

determine the species of origin of tissue culture cells (28). Strongly staining enzyme bands having migration rates compatible for the mouse were detected for lactate dehydrogenase, malate dehydrogenase, nucleoside phosphorylase, and glucose-6-phosphate dehydrogenase. Faulty staining bands comparable in position to rat were found on lactate dehydrogenase and nucleoside phosphorylase zymograms. Isozyme analysis confirms the finding by immunofluorescence that the majority population in the cell culture was mouse.

We do not yet know the potential proliferative lifespan of mouse mammary cells cultured in this way. Some of our early cultures were carried through 12 passages that included two clonings, and those cells still proliferated in culture. However, we did not record the seeding densities at each passage, and therefore do not know the number of doublings that the cells have undergone. More recently some new cultures have been carried to present date up through five passages and 30 doublings in cell number, and others through nine passages and 30 doublings. Both cultures still proliferated and formed numerous domes, and we will continue to subculture them to determine their longevity in culture.

DISCUSSION

Cells from normal mouse mammary gland can be cultured successfully when plated with irradiated rat mammary carcinoma cells of the LA7 line. The cells can be subcultured as long as new irradiated LA7 feeder cells are added at each passage, and the plating efficiency of the mouse mammary cells depends upon the density of feeder cells: the maximum efficiency was 14%. The longevity of these cells in culture is not yet known but the 30 doublings so far achieved represents about 85 generations of life for the cells now in culture at that stage as calculated by correcting for the plating efficiency (32). Thus the lifespan of these cells may approach the maximum achieved so far for epithelial cells of normal origin (11–13, 17, 33, 34).

This method differs from those developed for other epithelial cells in two respects. The growth medium is relatively undefined, containing 10% fetal calf serum instead of known hormones and growth factors, and the feeder cells are epithelial rather than fibroblastic in origin. Apparently our growth system contains the necessary nutrients or substitutes for them in some way. It is possible that the LA7 feeder cells produce some molecule(s) that is extruded into the medium that stimulates the proliferation of the normal mouse mammary cells, as in the growth systems developed for human mammary cells where conditioned medium from human fetal intestinal epithelial or hamster embryo fibroblast cells are necessary for stimulation of normal mammary cell growth (12, 16). It is also possible that the irradiated feeder cells produce a substrate more conducive than tissue culture plastic to normal mouse mammary cell growth in a similar way that the extracellular matrix from rat mammary glands stimulates the growth of rat mammary epithelial cells (5) or that collagen promotes proliferation of normal human and mouse mammary cells in culture (8, 9, 32). Additional advantage might be achieved by an extracellular matrix produced by the LA7 feeder cells in that an appropriate growth substrate for epithelial cells can reduce the nutritional requirements for growth (6, 36).

Two less well studied modes of growth stimulation by feeder cells may also operate here. The proliferation of cells may depend upon their shape or conformation, which the feeder cells may modify in a stimulatory way. A relationship between cell shape and growth has been shown for fibroblast cells (37). The other possible mode of growth stimulation may be a communication between the LA7 feeder cells and the normal mouse mammary cells. It is interesting in this regard that rat hepatocytes survive longer in culture and secrete albumin if co-cultured with a rat biliary epithelial cell line, and that the albumin secretion depends upon cell-to-cell contact with the biliary cells (38). Perhaps the same mechanism of cell contact operates to stimulate the mouse mammary epithelial cells to grow.

Whatever the mechanism of growth stimulation by the LA7 feeder cells, we know that some specificity exists as to the type of cell that will work successfully as a feeder. In our hands 3T3 fibroblasts did not stimulate mouse mammary cell growth, nor did four other epithelial cell lines (data to be presented elsewhere). So far we do not know whether species of origin of cells or tissue of origin determines the efficacy of cells as feeders, but an important clue to growth regulation must lie in this answer. Furthermore, the growth system reported here may be specific for the species (mouse) from which the cells of interest come or a function of the tissue (mammary). In addition, the method of tissue collection may also be important. For example, a minced whole mammary gland may contain a population of stem cells that differs from that obtained from milk.

The growth system presented here differs from most others not only in cell feeder type, but also in that the medium supplements used most often to stimulate growth for other cell types. Epidermal growth factor and cholera toxin (13, 17, 19, 21) do not stimulate growth of the mouse mammary cells when they are plated with LA7 feeder cells. Quite to the contrary, epidermal growth factor and cholera toxin are inhibitory to growth (data not shown). These facts lead us to suspect that the growth stimulus provided by the LA7 feeder system is qualitatively different from that in other reported growth systems, and we are presently pursuing an understanding of the nature of this growth stimulus.

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