Cancer Chemother Pharmacol (1981) 6:237–244

Cancer Chemotherapy and Pharmacology

Use of an Efficient Method for Culturing Human Mammary Epithelial Cells to Study Adriamycin Sensitivity

Helene S. Smith, Adeline J. Hackett, Sam Lan*, and Martha R. Stampfer

Peralta Cancer Research Institute, 3023 Summit Street, Oakland, Ca 94609
Donner Laboratory, University of California, Berkeley, California 94706, USA

Summary. *Techniques are described for isolating, cryopreserving, and culturing human mammary epithelial cells of both normal and malignant origin. The cells can be grown either in mass culture or as a clonal assay suitable for quantitating drug sensitivity. With this clonal assay plating efficiencies of 6%–41% were routinely obtained. We examined the response to adriamycin of five different primary carcinoma cultures from patients without prior drug therapy. We were able to detect heterogeneity in response to adriamycin among the breast carcinoma cultures as well as heterogeneity among subpopulations within a single carcinoma. The differences in adriamycin sensitivity were unrelated to growth rates in culture.*

Introduction

Techniques have been developed to evaluate chemotherapeutic drug sensitivity of dissociated cancer cells in agar suspension [5, 12]. Recent studies indicate that this in vitro assay correlates with patient response [1–3, 7, 12, 18, 19]. A number of technical difficulties prevent more widespread utilization of this agar assay technique. First, the plating efficiency is often lower than one colony per 10^4 cells plated; secondly, there are difficulties in obtaining single cell suspensions, particularly with scirrhus tumors; and thirdly, cryopreservation of primary tumor tissue has proven difficult.

We have developed techniques for growing human mammary cells in mass culture [16] or as colonies derived from single cells [6, 15]. These techniques solve the problem of cryopreservation and low plating efficiency for mammary specimens. In this paper, we summarize our techniques for culturing mammary epithelial cells, describe the highly efficient clonal assay system, and describe its use in the evaluation of five mammary carcinoma specimens for adriamycin sensitivity.

Reprint requests should be addressed to H. S. Smith
* *Present address:* Albert Einstein College of Medicine, Department of Surgery, New York, NY, USA

Materials and Methods

Specimen Material. All specimens were obtained as discard surgical material. Table 1 summarizes the donor histories.

Tissue Collection and Preparation. Tissue was obtained as discard material from reduction mammoplasties or mastectomies. Processing of tissue for separation of epithelial from stromal components was as described previously [16]. For the reduction mammoplasties, skin and grossly fatty areas were removed. The remaining mammoplasty and carcinomatous tissue was gently lacerated. The material was then digested with collagenase and hyaluronidase at 37° C with gentle rotation. The enzymatic reaction was terminated when microscopic examination showed epithelial clumps, termed organoids, free of attached stroma. These organoids were collected by filtration with polyester screen filters and stored frozen in multiple ampoules in liquid nitrogen. Depending upon the amount of tissue obtained, and the epithelial content of that tissue, 10–70 ampoules, containing approximately 100–250 organoids each, could be stored frozen from each reduction mammoplasty, while 3–6 ampoules could be stored from each carcinoma. To initiate experiments, an ampoule was quickly thawed and the organoids plated into multiple T-25 flasks (Corning).

Culture Procedures. The epithelial cells were grown in plastic petri dishes or flasks maintained at 37° C in a humidified CO_2 incubator. The medium (MM), which was a modification of enriched medium specifically designed for human mammary epithelial cells [16], consisted of the following: 30% Dulbecco's modified Eagle's medium (DME), 30% Ham's F-12, 15% conditioned medium from human fetal intestine epithelial cells, 74Int [14], 15% conditioned medium from human bladder epithelial cell line, 767B1 [14], 9% conditioned medium from human myoepithelial cell line 578Bst [4], 1% fresh newborn or fetal calf serum, 10 μg insulin/ml (Sigma, St Louis, Missouri), 5 ng epidermal growth factor/ml (Collaborative Research), 10^{-8} *M* triiodothyronine (Sigma), 10^{-9} *M* estradiol

0344-5704/81/0006/0237/$ 01.60

Table 1. Patient histories of specimen donors

Designation	Donor age (sex)	Pathology
Non-malignant tissues		
H3E	27 (Female)	Normal reduction mammoplasty
H97E	28 (Female)	Normal reduction mammoplasty
H163E	27 (Female)	Normal reduction mammoplasty
H164E	28 (Female)	Normal reduction mammoplasty
H165E	(Female)	Reduction mammoplasty with slight ductal papillary hyperplasia and slight lobular hyperplasia
H173P	45 (Female)	Mastectomy tissue peripheral to carcinoma; fibrocystic
Primary carcinomas		
H25T	76 (Female)	Infiltrating ductal carcinoma
H66T	55 (Female)	Invasive ductal carcinoma (lobular pattern)
H72T	71 (Female)	Invasive ductal carcinoma
H82T	49 (Female)	Infiltrating ductal carcinoma (comedo pattern)
H157T	33 (Female)	Infiltrating lobular carcinoma
H173T	45 (Female)	Infiltrating ductal carcinoma
H181T	29 (Female)	Invasive ductal carcinoma
Metastatic carcinomas		
145T	69 (Male)	Metastatic to hypodermus

(Sigma), 0.1 μg/ml hydrocortisone/ml (Sigma), 1 ng/ml cholera toxin/ml (Schwartz Mann), penicillin, and streptomycin. The medium was changed three times a week for stock cultures and every 48 h for experimental cultures. To subculture cells, total medium was removed, the dishes were washed once with STV (Ca^{2+}-, Mg^{2+}-free phosphate-buffered saline; trypsin 0.05%; versene 0.02%), and then small volumes of STV were added for varying periods of time at either 22° C or 37° C until the desired percentage of cells had detached. Cell counts were performed by counting trypan blue dye-excluding cells in a hemocytometer.

For the clonal assay, subconfluent primary or secondary cultures were trypsinized and 100 cells seeded onto 60-mm diameter dishes alone or containing 5×10^4 to 10^5 normal human skin fibroblasts (140Sk) from a patient with mammary carcinoma metastatic to hypodermis. For the irradiation studies, prior to addition of epithelial cells, medium was removed and dishes with fibroblasts were irradiated for 6 seconds at 24 in. from an ultraviolet germicidal light (General Electric #30T8, 30 W). Colony assays were fed twice weekly.

Assay of Adriamycin Toxicity

The day prior to the assay, PH140Sk fibroblasts were trypsinized and seeded at 5×10^4 cells/60-mm tissue culture dish in DME-F-12 plus 5% newborn calf serum. The next morning, the medium was drawn off and the uncovered dishes were irradiated as described above. A subconfluent culture of the epithelial specimen to be tested was trypsinized, dispersed to a single cell suspension, counted with a hemocytometer and plated onto the irradiated fibroblasts at 10^4, 10^3, or 200 cells/dish with MM medium. Twenty-four hours later, the medium was removed and MM medium with the desired drug concentration was added to each dish and the dishes incubated at 37° C. Four hours later, the medium was removed, and the dishes washed once with basal salts. Each dish was then refed with MM medium containing an additional 10^4 freshly trypsinized fibroblasts. Dishes were refed twice weekly until readily visualized colonies were present (usually within 5–10 days). Dishes were rinsed with phosphate-buffered saline, fixed with methanol, and stained with May-Grünwald-Giemsa. Adriamycin in lyophilized powder (Adria Laboratories, Inc.) was diluted with sterile physiologic saline at 1 mg/ml, aliquoted, and frozen at −20° C. For each experiment, a new ampoule was used.

Results

Culturing Human Mammary Epithelial Cells

To separate epithelial cells from the surrounding fibroblasts, minced tissue was treated with collagenase and hyaluronidase to digest stroma and basement membranes. The epithelial cells, which remained as clumps or organoids, were separated from dissociated stromal cells by filtration. The epithelial elements from normal tissue digests maintained ductular-alveolar-like structure [16]. In contrast, epithelial elements from digested tumor tissue were amorphous, irregularly shaped clumps (Fig. 1). The epithelial elements were aliquoted and successfully cryopreserved at this stage. In mass cultures, epithelial cells migrated from the organoids or clumps and underwent extensive proliferation. Figure 2 illustrates such an area of proliferation in a primary

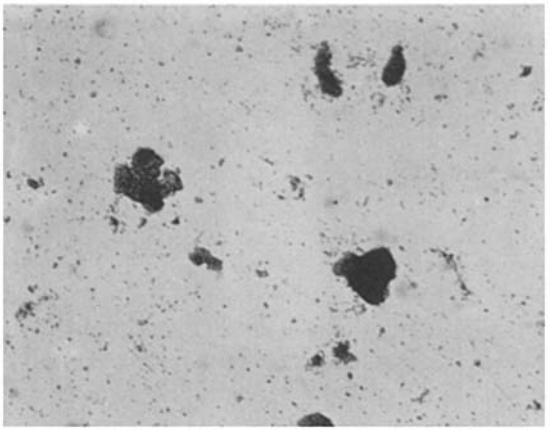

Fig. 1. Organoid preparation from human mammary carcinoma specimen. An infiltrating ductal carcinoma specimen (H185T) derived from a 72-year-old woman was minced and treated with collagenase and hyaluronidase overnight. 32×

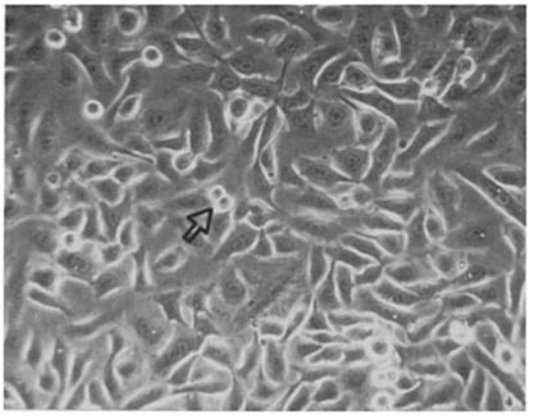

Fig. 2. Photomicrograph of proliferating epithelial cells in a primary culture derived from a mammary carcinoma. *Arrow* indicates a mitotic figure. 132×

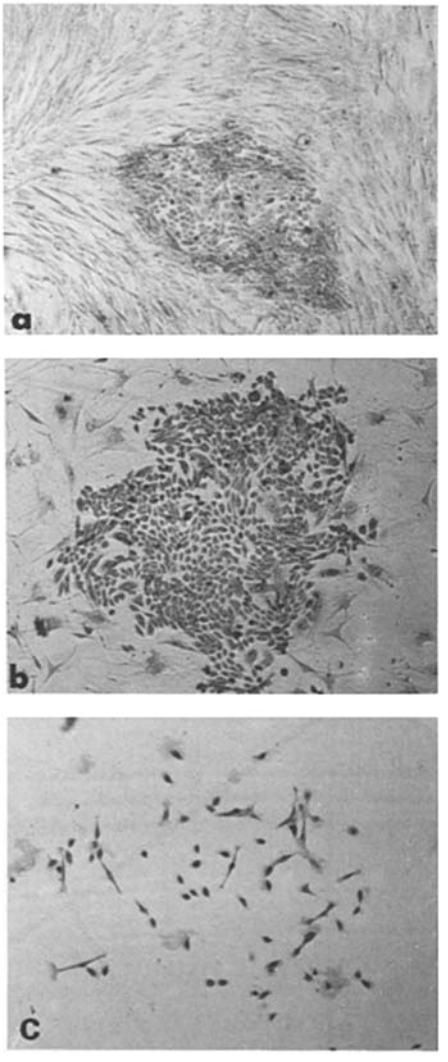

Fig. 3a–c. Photomicrographs of mammary epithelial colonies. Light micrograph of tumor cell specimen 157T on nonirradiated fibroblasts (**a**), irradiated fibroblasts (**b**), or plastic (**c**). 32×

carcinoma culture. When the primary cultures showed extensive mitotic activity at the periphery of each epithelial clump, mild trypsinization could be used to dissociate some of the replicating peripheral cells while leaving the central organoid intact. If the remaining organoid structures were refed with media, the process of migration and proliferation could be repeated a number of times. Cells from subconfluent, proliferating cultures could readily be passaged; if the primary cultures were allowed to become confluent, however, the cells were no longer capable of proliferation upon subculture.

A single cell suspension of proliferating cells could be plated together with fibroblasts at much lower densities than for mass cultures. Colonies which develop under these growth conditions provide a clonal assay [6, 15]. Figure 3 illustrates the effects of fibroblasts on colony development in the clonal assay. High plating efficiency was obtained when epithelial cells were plated with either irradiated or nonirradiated fibroblasts with formation of large compact colonies. In contrast, much smaller colonies and much lower plating efficiencies were seen when single cells were plated directly onto plastic. The plating efficiency of epithelial cells from different specimens on fibroblasts varied from 6%–41% (Table 2). The lower plating efficiencies were probably related to partial loss in proliferative potential because some areas of the dish had become confluent by the time the experimental cells were prepared.

Table 2. Efficiency of colony formation by mammary epithelial cells on fibroblasts[a]

Cell source	Colonies/100 cells plated
Non-malignant tissue[a]	
H97E	8
H3E	22
H163E	34
H164E	25
H165E	15
H173P	41
Primary carcinomas	
H157T	22
H82T	13
H25T	6
H173T	8
H72T	14
H66T	10
H181T	12
Metastatic carcinomas	
145T	6

[a] At 5–6 days the colonies contained at least 16–32 cells, while those at 12 days contained > 200 cells. Dishes were usually fixed on day 6–9. No difference in plating efficiency has been seen between scoring on days 6 and 12 [6, 15]

Table 3. Success rate in culturing human mammary epithelial cells

Source of tissues	No. of cultures capable of growth / Total no. of specimens received	
	Mass culture	Colony assay
Reduction mammoplasties	14/15	5/5
Primary carcinomas	9/10	7/7
Metastases		
To hypodermis	2/2	1/1
To lymph nodes	0/3	–
To pleura	0/1	–

Table 3 summarizes our success rate in culturing human mammary epithelial cells under clonal and mass culture conditions. Utilizing our most recent medium formulation (see *Materials and Methods* section), we compared the growth of all mammary specimens obtained from July through September, 1980. Over 90% of the cultures obtained from non-malignant tissues, primary carcinomas, and hypodermal metastases grew readily in mass culture for at least one passage. In every case tested, those specimens which grew in mass culture also grew in the clonal assay. The fact that the hypodermal metastases grew as well as the primary carcinomas excluded the possibility that only the non-malignant cells peripheral to carcinomas were capable of growth in culture. Although the sample size is small, so far we have been unsuccessful in growing lymph node or pleural metastases.

Table 4 summarizes the properties used to identify the cultured cells as mammary epithelial in origin. The cells were cuboidal in shape, morphologically characteristic of epithelial cells. Unlike endothelial cells, which are also cuboidal in morphology, the epithelial cells lacked anti-hemophilic factor VIII antigen. When the primary cultures were allowed to remain confluent for several weeks they formed dome-like structures, which are thought to be evidence of polarized secretory activity; the confluent cultures also produced multilayered ridges and tube-like structures. By electron microscopy, the cells showed typical epithelial type ultrastructural features including desmosome-like cell junctions, microvilli, and perinuclear bundles of tonofibrils. In addition, the cells were positive for the human mammary specific antigen prepared against milk fat globule, and showed a distinctive pattern of cell-associated fibronectin.

Table 4. Evidence for epithelial nature of cultured mammary cells

Property of cells	Reference
Cuboidal morphology	[16]
Makes domes, ridges	[16]
Ultrastructural evidence including junctional complexes, microvilli, and perinuclear tonofibril bundles	[16]
Absence of endothelial specific factor VIII antigen	Unpublished observation based on techniques described by Smith et al. [14]
Positive for mammary specific antigen	[9, 10, 15]
Abbreviated, punctate pattern of surface fibronectin	[14, 17]

Table 5. Sensitivity of cultured human mammary carcinomas to adriamycin

Tumor specimen	Drug concentration (ng/ml) resulting in	
	60% survival	< 0.1% survival
66T	1.0	100
72T	1.3	250
181T	2.0	500
173T	5.5	50
82T	15.0	5,000

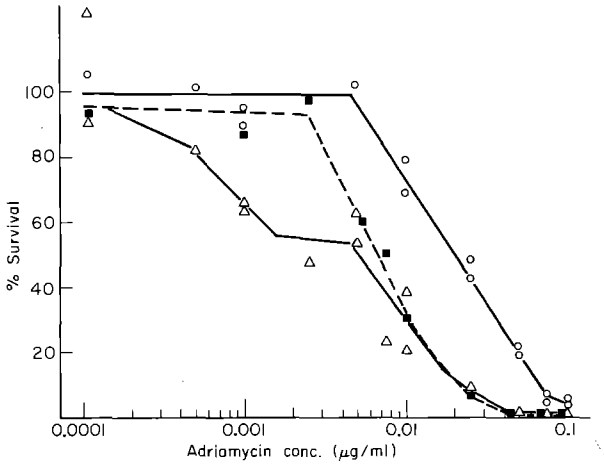

Fig. 4. Adriamycin toxicity of mammary carcinomas in second passage, measured by a clonal assay. —△—, specimen 72T; —■—, specimen 173T; —○—, specimen 82T

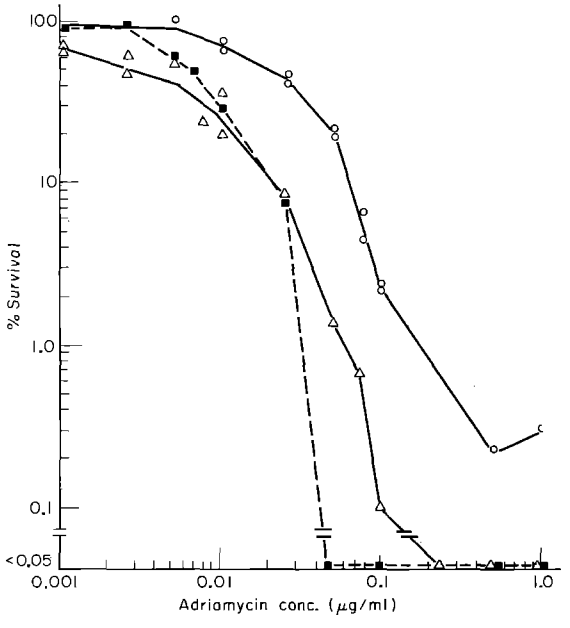

Fig. 5. Adriamycin toxicity of mammary carcinomas in second passages measured by a clonal assay. The data in Fig. 4 are plotted on a logarithmic scale for percent survival

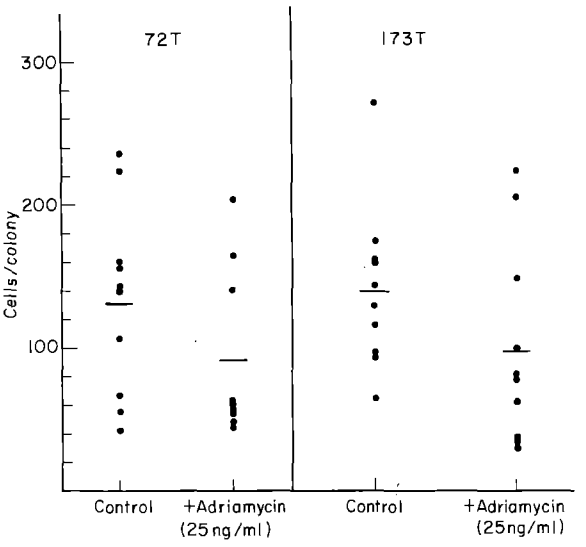

Fig. 6. Distribution of colony size as a function of prior drug treatment

Effects of Adriamycin on Cultured Human Mammary Carcinoma Cells

Table 5 summarizes the response to adriamycin of five different primary carcinoma, cultures from patients without prior drug therapy. The dose-response curves for three of these specimens are illustrated in Figs. 4 and 5. Figure 4 plots the percent survival on a linear scale. Plotting the data in this way accentuates the differences among the various cell populations within each tumor. One culture specimen, 72T, showed a biphasic response to adriamycin, with the more sensitive population being killed by approximately 12-fold less drug than a more resistant specimen, 82T. A third specimen, 173T, had an intermediate response to adriamycin. Figure 5 illustrates the same percent survival data plotted on a logarithmic scale to emphasize the dose response of the more resistant fraction of the cell population (i.e., those surviving after 90% of the clones had been killed). Although 40% of 72T cells were killed by less drug than 173T cells, the more resistant cell population in 72T required more drug than 173T cells to reduce the clonal survival to less than 0.1%. In Table 5 the drug concentrations are listed where both 60% and less than 0.1% of the colonies survived. While there was only a 15-fold range in drug concentration required for 60% survival, a 100-fold range in drug concentration was required to obtain less than 0.1% survival. In Table 5, the specimens are listed in order of increasing adriamycin concentration required for 60% survival. Heterogeneity in drug

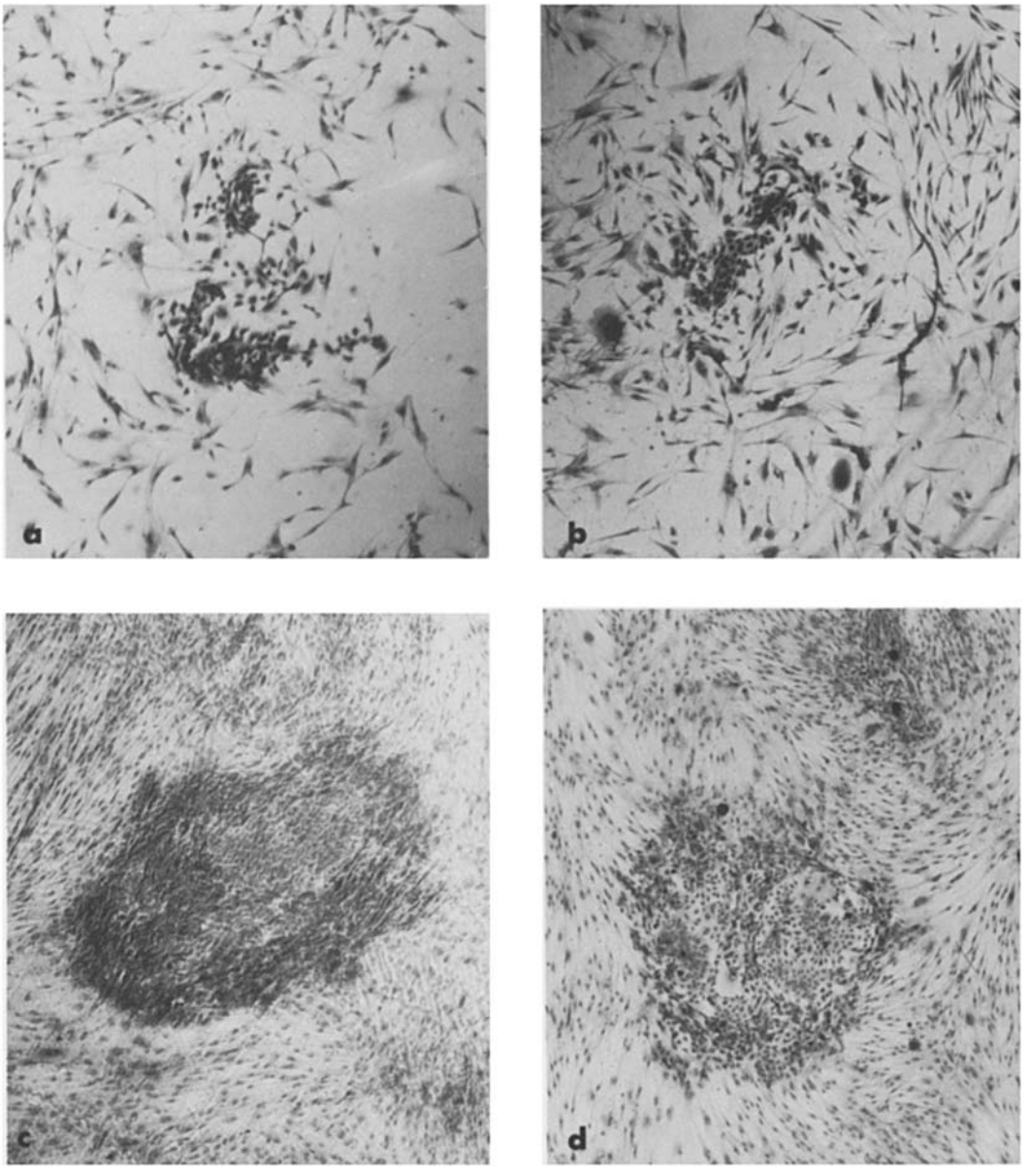

Fig. 7a–d. Morpohology of colonies as a function of drug treatment. **a** A colony of specimen 72T on an untreated control dish 6 days post seeding; **b** A colony appearing on a dish treated with 25 ng adriamycin/ml from the same experiment as in **a**; **c** A colony of specimen 173T on an untreated control dish 12 days post seeding; **d** A colony appearing on a dish treated with 10 ng adriamycin/ml from the same experiment as in **c**

response among the specimens is illustrated by the fact that the order among the cultures differed for 60% survival and less than 0.1% survival.

The effect of adriamycin on two cultures, 72T and 173T, was further examined by evaluating nine or ten randomly selected colonies for the number of cells per colony 6 days post seeding. Dishes treated with 25 ng adriamycin/ml and untreated controls were examined (Fig. 6). In the untreated dishes, the mean number of cells per colony was identical for the two tumor specimens, indicating that their differential in drug response was unrelated to growth rate. In both cases, the mean number of cells per colony was lower in drug-treated dishes; however, some of the colonies surviving drug treatment were quite large. As illustrated in Fig. 7, some colonies after drug treatment were indistinguishable from untreated controls at either 6 or 12 days post seeding.

Discussion

We have developed techniques for isolating and culturing human breast epithelial cells of both normal and malignant origin. These studies suggest possible solutions to some of the technical problems plaguing stem cell assays. After enzymatic digestion, we isolate the epithelial tissue as clumps. These clumps are separated from fibroblasts which have been dissociated to single cells by the enzyme digestion. When the epithelial cells are maintained as clumps, they can be readily cryopreserved. Rather than trying to dissociate the clumps prior to subculturing, which has proven difficult [13], we allow proliferation to commence from the plated epithelial clumps. At this stage, the cells can be readily trypsinized and dissociated into a single cell suspension suitable for drug sensitivity studies. With these techniques, we obtain plating efficiencies from 6%–40%, significantly higher than other assays. In addition, sufficient cells can be obtained for numerous drug assays, while cryopreservation provides a cell pool for reproducibility and convenience.

Utilizing this approach, we have measured the adriamycin sensitivity of five breast carcinoma specimens from previously untreated donors. We were able to detect heterogeneity in response to adriamycin both among the breast carcinoma cultures and among subpopulations within a single carcinoma. These results raise many exciting basic research questions relating both to the source of heterogeneity and to potential clinical applications.

In the classic stem cell assay [12], tumor cells are treated as soon after removal from the patient as possible. In the assay that we have designed, tumor cells are first allowed to enter the proliferative state and then treated with the drug. Thus, we are not concerned with size of the tumor's in vivo proliferative pool at the time of resection. Rather, we are asking whether there are inherent differences in adriamycin sensitivity among proliferating tumor cell populations. Clearly, studies are needed to evaluate clinical correlations by this approach. The data of Rosenblum et al. [11] are of significance: these authors found that the drug sensitivity was identical for glioma cells tested immediately after resection and after subculture.

Even if clinical correlations are found, further work on the cell biology of the system will be required before it can be widely used as a clinical test. Although we have successfully cultured all of the primary mammary carcinomas and hypodermal metastases, we have been unsuccessful in culturing mammary carcinomas metastatic to lymph nodes or pleura. Methods should be developed to grow specimens from these metastatic sites, since they are likely candidates for in vitro drug sensitivity tests. Secondly, the medium that we developed for mammary epithelial cells does not support extensive proliferation of cells from other organ systems (unpublished observations). Therefore, more study will be needed to optimize culturing of other malignancies. Finally, the medium that we developed requires conditioning factors from other epithelial and myoepithelial cell lines. These lines are not permanently established, and hence they have a finite lifespan. Therefore it is not practical to use this source of growth factor for large-scale commercial testing. More work will be needed either to develop a medium that no longer requires conditioning or to develop alternative sources for the growth factor provided by the epithelial cell lines.

Acknowledgements. This manuscript is dedicated to Mrs. Bernice Cohen Sterbakov. We wish to thank Dr. Emil Frei and Dr. Arthur Pardee (Sidney Farber Cancer Center, Boston, Mass.) for helpful discussions and encouragement in the early phases of the work. We also wish to thank Mr. Alan J. Hiller for excellent technical assistance. This work was supported by the Peralta Hospital Association, the Peralta Medical Foundation, National Cancer Institute Grants CA25630, CA25418, CA24844, and American Cancer Society Grant CD-26.

References

1. Alberts DS, Salmon SE, Chen H, Surwit SG, Moon TE (1980) Correlative and predictive accuracy of the human tumor stem cell assay for anticancer drug activity in ovarian cancer patients. Proc Am Soc Clin Oncol 16: 431
2. Alberts DS, Chen HSG, Salmon SE, Surwit EA, Young L, Moon TE (1981a) Chemotherapy of ovarian cancer directed by the human tumor stem cell assay. Cancer Chemother Pharmacol 6: 279–285
3. Alberts DS, Salmon SE, Chen HSG, Moon TE, Young L, Surwit EA (1981b) Pharmacologic studies of anticancer drugs with the human tumor stem cell assay. Cancer Chemother Pharmacol 6: 253–264
4. Hackett AJ, Smith HS, Springer EL, Owens RB, Nelson-Rees WA, Riggs JL, Gardner M (1977) Two syngeneic cell lines from human breast tissue: One aneuploid mammary epithelial (Hs578T) and one diploid myoepithelial (Hs578Bst). J Natl Cancer Inst 58: 1795–1806
5. Hamburger AW, Salmon SE (1977) Primary bioassay of human tumor stem cells. Science 197: 461–463
6. Lan S, Smith HS, Stamper MR (1981) Clonal growth of normal and malignant human breast epithelia. J Surg Oncol (in press)
7. MacKintosh FR, Evans TL, Sikic BI (1981) Methodologic problems in clonogenic assays of spontaneous human tumors. Cancer Chemother Pharmacol 6: 205–210
8. Peehl DM, Ham RG (1980) Growth and differentiation of human keratinocytes without a feeder layer on conditioned medium. In Vitro 16: 516–525
9. Peterson JA, Buehring GC, Taylor-Pappadimitriou J, Ceriani RL (1978) Expression of human mammary epithelial (HME) antigens in primary cultures of normal and abnormal breast tissue. Int J Cancer 22: 655–661

10. Peterson JA, Bartholomew JG, Stampfer M, Ceriani RL (1980) Quantitative changes in expression of human mammary epithelial (HME) antigens in breast cancer as measured by flow cytofluorimetry. J Exp Biol
11. Rosenblum ML, Dougherty DV, Reese C (1981) Potentials and possible pitfalls of human stem cell analysis. Cancer Chemother Pharmacol 6: 227–235
12. Salmon SE, Hamburger AW, Soehnlen BS, Durie BGM, Alberts DS, Moon TE (1978) Quantitation of differential sensitivity of human-tumor stem cells to anticancer drugs. N Engl J Med 298: 1321–1327
13. Slocum HK, Pavelic ZP, Kanter PM, Nowak NJ, Rustum YM (1981) The soft agar clonogenicity and characterization of cells obtained from human solid tumors by mechanical and enzymatic means. Cancer Chemother Pharmacol 6: 219–225
14. Smith HS, Hackett AJ, Riggs JL, Mosesson ML, Walton JR, Stampfer MR (1979) Properties of epithelial cells cultured from human carcinomas and nonmalignant tissues. J Supramol Struct 11: 147–166
15. Stampfer MR, Allows PC, Hackett AJ (1980) Growth of normal human mammary cells in culture. In vitro 16: 415–425
15a. Smith HS, Hackett AJ, Lans, Stampfer R (1981) Use of an efficient method for culturing human mammary epithelial cells to study adriamycin sensitivity. Cancer Chemother Pharmacol 6: 237–244
16. Stampfer MR, Vlodavsky I, Riggs JL, Smith HS (1981) Fibronectin production by human mammary epithelial cells in culture. J Natl Cancer Inst (in press)
17. Von Hoff DD (1980) Clinical correlations of drug sensitivity in tumor stem cell assay. Proc Am Assoc Cancer Res 71: 134
18. Von Hoff DD, Cowan J, Harris G, Reisdorf G (1981) Human tumor cloning: feasibility and clinical correlations. Cancer Chemother Pharmacol 6: 265–271

Received June 11, 1981