PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **174**, 276–283 (1983)

Sequential Study on the Influence of Adriamycin on Cell Proliferation and Ultrastructure of Cultured Mammary Tumor Cells[1] (41737)

AHMAD R. SAFA, MICHAEL T. TSENG, AND RICKY J. BALLOU

Department of Anatomy and the Brown Regional Cancer Center, University of Louisville Health Sciences Center, Louisville, Kentucky 40292

Abstract. The time-dependent cytocidal and growth inhibitory effects of Adriamycin (ADM) on monolayer cultures of 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced mammary tumor cells were analyzed. The inhibitory effect on cell proliferation was assessed by colony formation in soft agar. Growth inhibition and [^{3}H]thymidine labeling indices clearly demonstrate a dose-dependent antimitotic and cytotoxic effect of the drug. At low concentrations (10^{-9}–10^{-8} *M*), 90–100% of cells survived 24-hr exposure. At a higher concentration (10^{-5} *M*), 75–80% of cells survived after 8-hr exposure; by 72 hr only 20–30% of the cells remained. Autoradiographic examination of the pulse-labeled cultures demonstrated no change in the proportion of cells in S-phase during the first 4 hr of treatment. Subsequently DNA synthesis was completely abolished and remained inhibited for the duration of the experiment (72 hr). Clonogenic assay revealed a complete arrest of growth in cells exposed to 10^{-5} *M* ADM and greater than 60% inhibition of cell proliferation at 10^{-7} *M*. Ultrastructural changes were not observed in cells during the first 4 hr of treatment; however, after 8 hr most surviving cells exhibited alterations in nuclear chromatin. The surviving cells also showed mitochondrial degeneration, myelin body formation, and vacuolization of the endoplasmic reticulum. This study shows the potential usefulness of the primary culture system in drug evaluation. In addition, serial observation of the effects of ADM revealed a cell subpopulation of the primary culture with differential sensitivity to the drug.

Adriamycin (ADM), an anthracycline antibiotic often used as a single chemotherapeutic agent, has a response rate of approximately 35% in the treatment of human breast cancer (1, 2). The cell cycle-nonspecific nature of this drug makes it particularly useful in the treatment of solid tumors containing predominantly slow-growing cells. Considerable evidence indicates that ADM, like other intercalating agents, induces single-strand breaks in the DNA of mammalian cells (3–5), binds to DNA and inhibits DNA synthesis (4, 6), and thus exhibits a cytotoxic effect (7, 8).

Cultured cell lines maintained in defined media should prove useful in examining the mechanism of action of specific therapeutic agents. However, in the clinical setting tumors rarely consist of a single cell type nor exist in a constant and defined milieu. An alternative way to evaluate therapeutic agents would be to assess the efficacy of drugs on primary cultures from tumor biopsies. In a previous report (9) we demonstrated that a tumor cell primary culture system may be useful in predicting the response of the original tumor to therapy. In this study, a similar system has been used to evaluate the time-dependent effects of ADM on the growth kinetics and ultrastructure of cultured DMBA-induced mammary tumor cells.

Materials and Methods. *Tumor induction and cell culture conditions.* Mammary tumors were induced in 50-day-old female Sprague–Dawley rats by gastric intubation of 5 mg DMBA in a 15% fat emulsion (Upjohn Co., Kalamazoo, Mich.). Eighty-five percent of treated rats developed mammary tumors within 6 to 10 weeks. Tumors were surgically removed and, after enzymatic dispersion of tumor tissue (9), primary cultures were prepared by plating 8×10^5 cells per well in multiwell culture plates (16.4 mm diameter, Costar Plastics, Cambridge, Mass.). Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum, hormones, and anti-

[1] Supported in part by American Cancer Society Grant DPT-100A and a grant from the Graduate School, University of Louisville, Ky.

0037-9727/83 $1.50

biotics was used to maintain the culture (9). Cells were incubated for 4–6 days at 37°C in a humidified atmosphere of 5% CO_2–95% air.

Drug treatment, [³H]thymidine labeling indices, and colony forming assay. To establish dose–response curves, cells in the log phase of growth were treated with ADM at concentrations between 10^{-4} and 10^{-9} *M*. Twenty-four hours later the ADM-containing medium was removed, the cultures were washed three times in Ca^{2+}-Mg^{2+}-free phosphate-buffered saline and attached cells were removed by incubation of the cultures in a trypsin–Versene mixture (9). Cell numbers were determined using a Coulter counter. The viability of cells was checked by the trypan blue dye exclusion test. The number of viable attached cells after treatment with different concentrations of ADM was expressed as a percentage of the control value.

For sequential analysis of the effects of the drug, cultured cells were treated with 10^{-5} *M* ADM for 1, 2, 4, 8, 24, or 72 hr. After washing individual wells three times in Ca^{2+}-Mg^{2+}-free phosphate-buffered saline, the remaining attached cells were freed from the substrate by use of a trypsin–Versene mixture (M. A. Bioproducts, Walkersville, Md.). Cells were counted and the number expressed as a percentage of the control value.

Thymidine labeling indices of each culture, which reflect the proportion of cells in S-phase at a given time, were determined autoradiographically. After treatment with ADM, cells were pulsed with 0.5 ml of 1 μCi [³H]thymidine per well for 30 min before the scheduled termination of the experiment. After thymidine administration, cells were washed three times with Ca^{2+}-Mg^{2+}-free phosphate-buffered saline and fixed with 10% neutral buffered Formalin. After dehydration through graded ethanols (30–100%), 5 min each, the bottoms of the culture wells were cut out and mounted on glass slides. The slides were then coated with diluted Kodak NTB-2 nuclear track emulsion and stored at 4°C in a light-proof box with desiccant. After development, the slides were stained with hematoxylin and eosin. A minimum of 3000 cells were counted and the number of S-phase cells after various treatments was expressed as a percentage of the control value.

For the cell colony forming assay, the single cell suspension was adjusted to a final concentration of 5×10^5 cells/ml in the presence of 10^{-5} or 10^{-7} *M* ADM. Cells were incubated with or without drug for 1 hr at 37°C. The colony forming assay used in this study has been described previously (10, 11). Briefly, cells to be tested were suspended in 0.3% agar (Difco Laboratories, Detroit, Mich.) in Connaught Medical Research Laboratories Medium 1066 (CMRL 1066) supplemented with 15% horse serum (M. A. Bioproducts, Walkersville, Md.), 0.5 μg/ml insulin, 1 μg/ml prolactin, 10,000 units/ml penicillin, 10,000 units/ml streptomycin, and 25 μg/ml fungizone. One milliliter of this suspension was pipetted into a 35-mm petri dish containing 1 ml of 0.5% Difco Bacto agar in enriched (10% fetal calf serum, 0.5 μg/ml insulin, 1 μg/ml prolactin, 10,000 units/ml penicillin, 10,000 units/ml streptomycin, and 25 μg/ml fungizone) McCoy's 5A medium. Cultures were set up in triplicate and incubated at 37°C in a humidified atmosphere of 5% CO_2–95% air.

Colonies containing 30 or more cells appeared at 14–21 days, at which time the number of colonies on control and treated plates was counted using an inverted-phase microscope. The number of colonies in the three plates for each drug concentration was averaged and the standard deviation from the mean was determined to obtain one data point. The number of colonies surviving at each drug concentration was expressed as a percentage of the control value.

Electron microscopy. For ultrastructural analysis, cells were collected and pelleted in 1.5-ml conical microcentrifuge tubes, fixed, and processed for electron microscopy as described previously (12). Thin sections were cut using an MT-2 ultramicrotome, stained with uranyl acetate and lead citrate, then examined with a Philips 300 transmission electron microscope. To establish the effect of ADM on the ultrastructure of the cell population, a minimum of 500 cells from control and treated samples were examined. Cells showing a substantial portion of nucleus and cytoplasm were counted and tabulated on the basis of the presence or absence of cytoplasmic vacuoles.

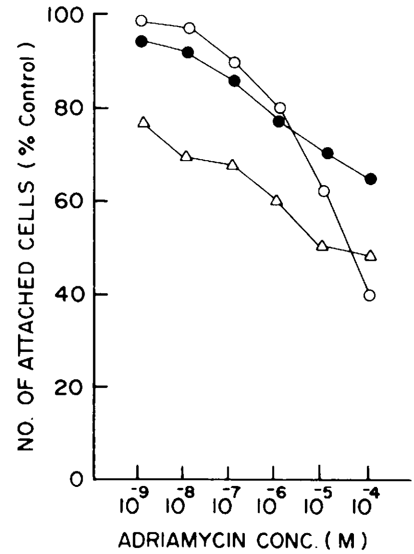


FIG. 1. Dose-response curves of primary cell cultures from DMBA-induced mammary tumors. Each symbol (○), (●), and (△), represents the average response of triplicate wells of primary cultures derived from a single tumor at different concentrations of ADM. Standard deviation from the mean for each point is less than 10%.

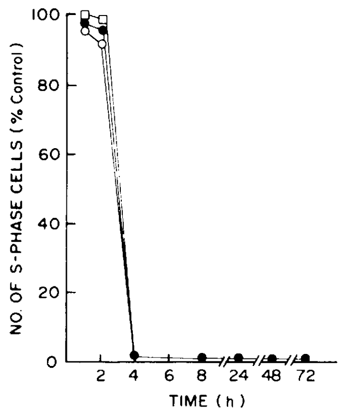


FIG. 3. Sequential effect of 10^{-5} *M* ADM on [^{3}H]thymidine incorporation on the corresponding cultures as illustrated in Figs. 1 and 2. After 4-hr treatment with 10^{-5} *M* ADM, DNA synthesis completely stops. Each point shows the percentage of S-phase cells compared to the control for a minimum of 3000 cells from each culture well. Each symbol (□), (●), and (○), represents the average response of three plates of cells obtained from a single tumor. Standard deviation from the mean for each point is less than 10%.

Results. *Growth inhibition and sequential effects of adriamycin treatment.* An average of 7.4×10^7 viable cells were recovered from 1 g of wet mammary tumor tissue. After cell dispersion, viability was found to be 85–95%. Considerable variation in the growth rates of primary cultures was observed. Doubling times of these cultures ranged from 40–75 hr with a mean of 52 hr. The cells reached complete confluency 4–5 days after plating. The dose-response curves of three primary cultures are presented in Fig. 1. At low drug concentrations (10^{-8}–10^{-9} *M*), 90–100% of cells survived 24 hr of treatment. Successive increases in ADM concentration caused a gradual decrease in cell survival. The most effective concentrations of ADM were between 10^{-4} and

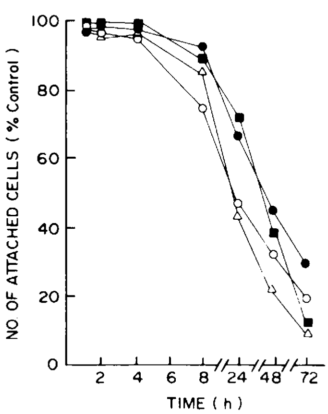
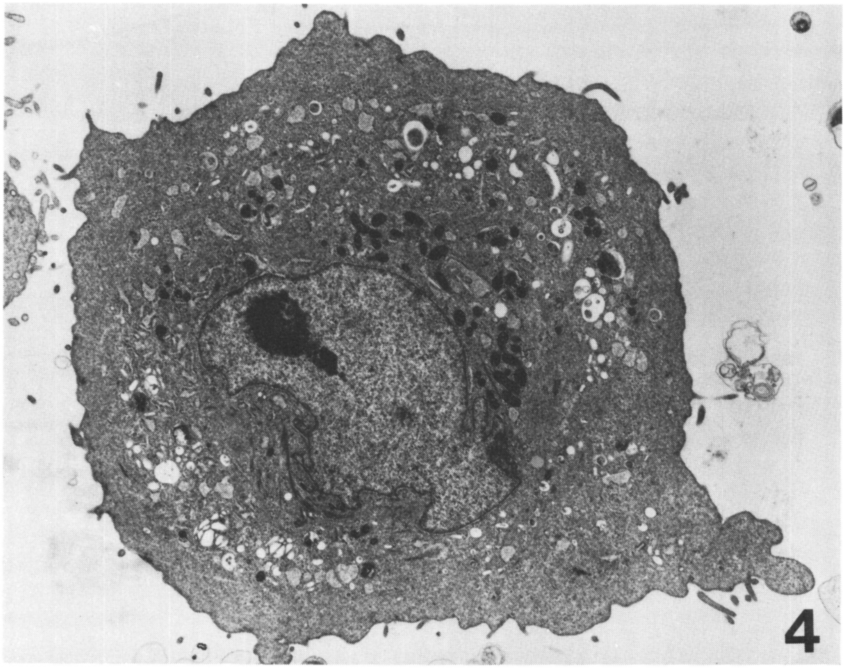


FIG. 2. Time-dependent response of primary cell cultures from DMBA-induced mammary tumors treated with 10^{-5} *M* Adriamycin for 72 hr. Each symbol (■), (●), (○), and (△), represents the average response of triplicate wells of primary cultures derived from a single tumor at different concentrations of ADM. Standard deviation from the mean for each point is less than 10%.

TABLE I. COLONY FORMING ASSAY

ADM concentration	Number of colonies $\bar{X}$	SD	Percentage of control
Control	301.6	25.3	
10^{-7} *M*	110.0	21.6	36
10^{-5} *M*	0	0	0

Note. $\bar{X}$ (mean) and standard deviation (SD) were determined in three dishes and triplicate experiments.



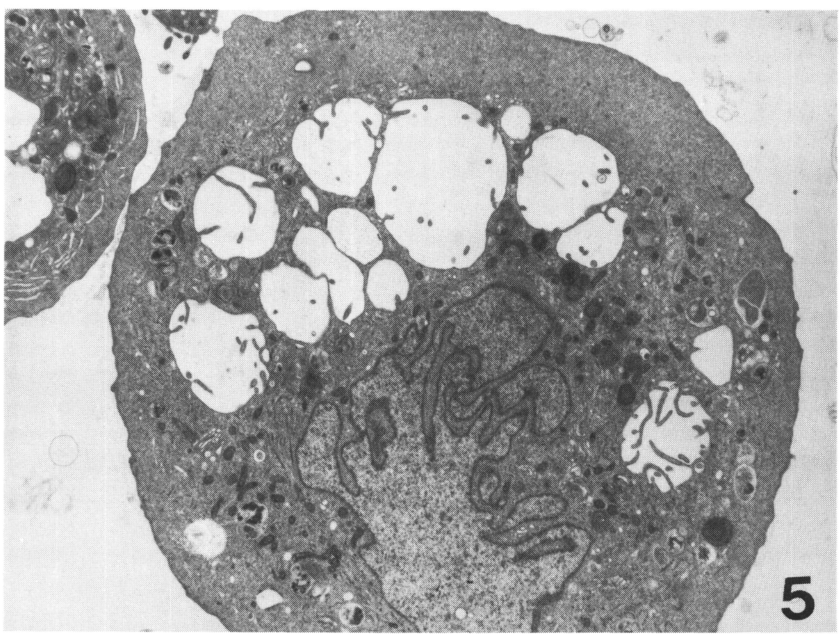
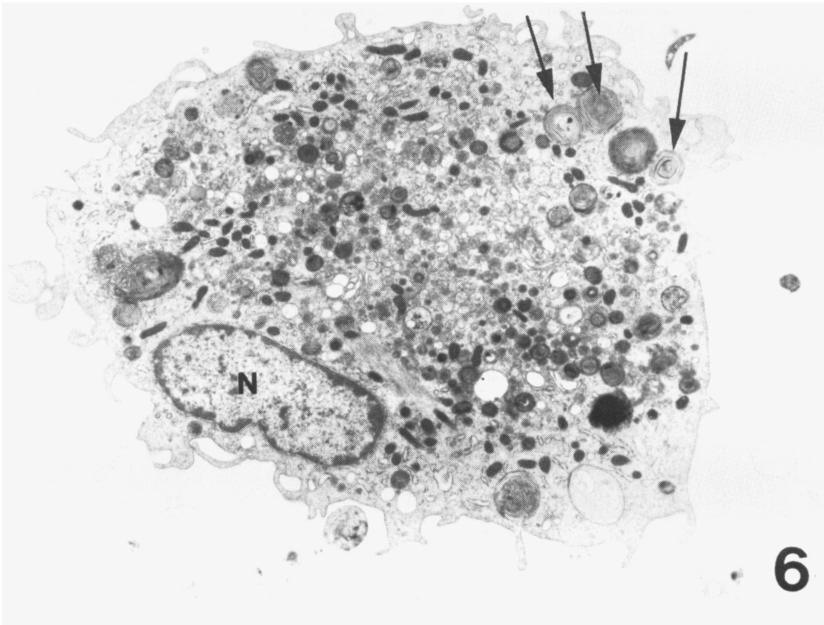


FIG. 4. A nonvacuolated DMBA-induced mammary tumor cell. Its cytoplasm tends to be more electron-dense than that of the vacuolated cell. ×3900.

FIG. 5. Numerous cytoplasmic vacuoles are the distinguishing features of vacuolated tumor cells. ×3640.

10^{-6} *M*. Following 24-hr treatment with 10^{-5} *M* ADM, about 50% of cultured cells remained. Comparison of cell numbers at the initiation and termination of experiments in treated and control samples revealed 20–30% cell death at 10^{-5}–10^{-4} *M* ADM.



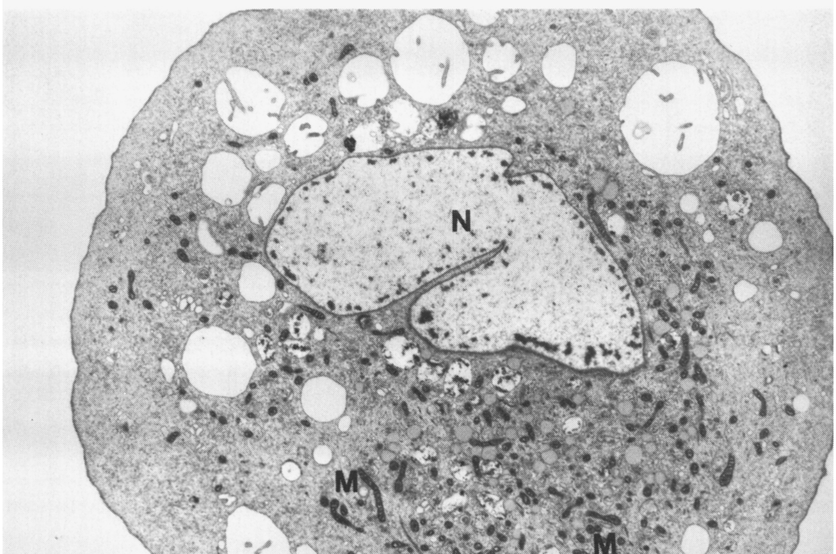


FIG. 6. Margination of nuclear chromatin (N) and marked accumulation of myelin bodies (arrows) represent the Adriamycin-induced lesions in nonvacuolated cells. ×4900.

FIG. 7. Marked nuclear (N) damage is shown in the vacuolated cell. Increased mitochondrial (M) density is also apparent. ×6800.

Using 10^{-5} M as the standard working concentration, cultures were exposed to ADM for up to 72 hr with cell removal at the specified intervals. Little or no cytotoxic effect was observed during the first few hours of treatment. After 8-hr treatment, 75–95% of cells remained after ADM exposure. The cell number declined to about 50% of the control value after 24 hr; by 72 hr only 20–30% of the cells remained (Fig. 2).

Autoradiographic evaluation of the proportion of cells in S-phase indicated that no change in DNA synthesis occurred during the first 4 hr of treatment (Fig. 3). However, by 8 hr post-treatment, DNA synthesis was completely arrested (Fig. 3). DNA synthesis remained inhibited for the remainder of the experiment (72 hr).

Colony forming assay revealed a sharp decrease in the number of cell colonies after exposure of cells to 10^{-7} M ADM for 1 hr (Table I). The number of colonies at this concentration was only 36% of the control value. At 10^{-5} M, the drug completely suppressed clonogenicity.

Ultrastructural changes in adriamycin-treated cultured mammary tumor cells. Based upon the presence or absence of intercellular vacuoles, two cell types were distinguished. Both cell types appear to have an epithelial origin since both show scattered tonofilaments. A relatively opaque cytoplasm coupled with the absence of large vacuoles distinguished nonvacuolated cells from vacuolated cells (Figs. 4, 5).

Cells exposed to ADM for up to 4 hr appeared virtually undisturbed. Slight margination of heterochromatin was the only apparent nuclear response. After 8 hr the nuclei of most surviving cells showed a change in chromatin distribution. Aggregates of heterochromatin persisted near the nuclear envelope and the remaining nucleoplasm was electron-lucent and finely particulate. This nuclear derangement occurred in an all-or-none fashion and was no greater at 24 hr than at 8 hr of drug exposure. A slight increase in lysosomal activity, evidenced by accumulation of membrane-bound vesicles containing electron-opaque particles, constituted the sole cytoplasmic alteration in cells surviving ADM treatment (Figs. 6, 7). Among cellular organelles, mitochondrial degeneration, vacuolization of the endoplasmic reticulum, and myelin body formation were frequently encountered.

The proportion of vacuolated versus nonvacuolated cells in a given culture was not altered during the first 4 hr of drug exposure.

TABLE II. PERCENTAGE OF VACUOLATED CELLS IN UNTREATED CULTURES AND IN CULTUES EXPOSED TO 10^{-5} M ADRIAMYCIN FOR UP TO 24 hr

Exposure time (hr)	Percentage vacuolated cells*	SD*
0	58.2	4.6
1	53.5	8.5
2	56.0	7.6
4	52.0	11.2
8	45.6	8.7
24	31.8	6.2

* Percentage of vacuolated cells and standard deviation from the mean (SD) were obtained for a minimum of 500 cells from control and treated samples from three examined tumors.

Twenty-four hour exposure to ADM appeared to affect the vacuolated cells more than the nonvacuolated cells since only 31.8% of the surviving cell population is vacuolated compared with 58.2% of the control (Table II).

Discussion. Our data clearly show that ADM exerts both cytocidal and growth inhibitory effects on cultured DMBA-induced mammary tumors. Dose–response curves from these cultures indicate that both effects are progressively accentuated as the drug concentration increases from 10^{-9} to 10^{-4} M. These findings are consistent with those in the literature (13). The colony forming assay used to assess drug-induced reproductive sterility in several systems (10, 11, 14) supports our thymidine labeling index data and clearly demonstrates complete suppression of cell proliferation at 10^{-5} M ADM. The basic mechanism for both effects is presumably the interaction of ADM and DNA with subsequent inhibition of DNA and RNA synthesis (3, 5, 15). In addition to inhibition of DNA synthesis and subsequent cell proliferation, ADM may suppress growth in part by altering the balance of cAMP and cGMP (16, 17). During exposure of cells to ADM for progressively longer periods, no significant change in the number of surviving cells is seen in the early hours of drug treatment. After 4-hr treatment with 10^{-5} M ADM, DNA synthesis was completely arrested in cultured cells; however, the cytocidal effect of the drug did not appear until at least 8 hr of exposure. After 24-hour treatment with 10^{-5} M ADM about 50% of cells survived; after 72 hr, only 10–15% survived. Thus, the growth inhibitory effect precedes the cytocidal effect.

ADM-induced subcellular changes include accumulation of concentric membrane-bound figures (myelin bodies) in cytoplasm, fragmentation and segmentation of chromatin in nuclei, vacuolization of the endoplasmic reticulum, and fine structural changes in mitochondria. These cellular lesions were only visible after 8-hr exposure of cells to the drug and are comparable to those seen in myocytes damaged by ADM (18). Comparable nuclear alterations have been reported in nuclei of myocardial and hepatic cells after exposure to ADM and daunorubicin *in vivo* (19, 20). These nuclear lesions result from the intercalation of drug into the DNA molecule, subsequent inhibition of DNA synthesis, and uncoiling of chromatin.

Study of the time-dependent growth inhibitory, cytocidal, and ultrastructural effects of ADM on DMBA-induced mammary tumors has enabled us to show a selective influence of the drug on vacuolated cells and suggests a differential sensitivity of these cells to the drug treatment. Ultrastructurally, vacuolated cells appeared to be better-differentiated than nonvacuolated cells. It may be inferred that ADM preferentially affects better-differentiated cells. Although the nature of this effect is not known, recent reports from our laboratory, using Ficoll gradient-enriched subpopulations of the cells, confirmed that the activity of lactate dehydrogenase and peroxidase in vacuolated cells is much lower than in nonvacuolated cells (21, 22). In contrast, a positive correlation between the level of estrogen receptor protein and vacuolization was encountered (23). Thus the two cell types appear to be ultrastructurally distinct with differential sensitivity to the drug. The underlying processes involved remain to be discovered.

The authors wish to thank Ms. Mary D. McCauley for her assistance in the preparation and editing of this manuscript.

1. Carter SK. Adriamycin—A review. J Natl Cancer Inst **55**:1265–1274, 1975.
2. Tormey DC. Adriamycin (NSC-123127) in breast cancer: An overview of studies. Cancer Chemother Rep **6**:319–327, 1975.
3. Lee YC, Byfield EJ. Induction of DNA degradation *in vivo* by Adriamycin. J Natl Cancer Inst **57**:221–224, 1976.
4. Merriwether WD, Bachur NR. Inhibition of DNA and RNA metabolism by daunorubicin and Adriamycin in L1210 mouse leukemia. Cancer Res **32**:1137–1142, 1972.
5. Schwartz HS. Biochemical action and selectivity of intercalating drugs. Advan Cancer Chemother **1**:1–60, 1979.
6. Kanter PM, Schwartz HS. Quantitative model for growth inhibition of human leukemia cells by antitumor anthracycline derivatives. Cancer Res **39**:3661–3672, 1979.
7. Eichholtz-Wirth H. Dependence of the cytotoxic effect of Adriamycin on drug concentration and exposure time *in vitro*. Brit J Cancer **41**:886–891, 1980.
8. Morasca L, Fogar-Ottaniano EG, Garattini S. Time dependent cytotoxicity of Adriamycin and daunomycin in primary cultures of normal and neoplastic mammary glands. Eur J Cancer **12**:107–111, 1976.
9. Safa AR, Tseng MT. Doxorubicin-induced ultrastructural and growth changes in primary cultures of 7,12-dimethylbenz[*a*]anthracene-induced mammary tumors. J Natl Cancer Inst **70**:422–450, 1983.
10. Hamburger AW, Salmon SE, Kim MB, Trent JM, Sachnian BJ, Alberts DS, Schmidt HJ. Direct cloning of human ovarian carcinoma cells in agar. Cancer Res **38**:3438–3444, 1978.
11. Hamburger AW, Salmon SE. Primary bioassay of human tumor stem cells. Science **197**:461–463, 1977.
12. Fink SM, Tseng MT. Effects of thiotepa on primary cultures of DMBA-induced mammary tumors of rats: Kinetic and ultrastructural study. Brit J Cancer **44**:762–765, 1981.
13. Landolph JR, Bhatt RS, Telfer N, Heidelberger C. Comparison of Adriamycin- and ouabain-induced cytotoxicity and inhibition of ^{86}rubidium transport in wild-type and ouabain-resistant C3H/10T1/2 mouse fibroblasts. Cancer Res **40**:4581–4588, 1980.
14. Buick RN, Stanisic TH, Fry SE, Salmon SE, Trent JM, Krasovich P. Development of an agar–methylcellulose assay for cells in transitional cell carcinoma of the human bladder. Cancer Res **39**:5051–5056, 1979.
15. DiMarco A. Adriamycin (NSC-123127): Mode and mechanism of action. Cancer Chemother Rep **6**:91–106, 1975.
16. Cho-Chang YS, Clair T, Bodwin JS, Berghoffer B. Growth arrest and morphological change of human breast cancer cells by dibutyryl cyclic AMP and L-arginine. Science **214**:77–97, 1981.
17. Witfield JE, Boynton AL, MacManus JP, Rixon RH, Walter PR, Armato U. The positive regulation of cell proliferation by a calcium–cyclic AMP control cou-

plet. In: Abou-Sabé M, ed. Cyclic Nucleotides and Regulation of Cell Growth. Straudsburg, Pa., Daviden, Hutchingson and Ross, p97, 1977.

18. Young DM. Pathologic effects of Adriamycin (NSC-123127) in experimental systems. Cancer Chemother Rep **6**:159–175, 1975.
19. Buja LM, Ferrans VJ, Mayer RJ, Roberts WC, Henderson HS. Cardiac ultrastructural changes induced by daunorubicin therapy. Cancer **32**:771–778, 1973.
20. Buja LM, Ferrans VJ, Rabson AS. Unusual nuclear alterations. Lancet **1**:402–403, 1974.
21. Safa AR, Tseng MT. Lactate dehydrogenase activity in cell subpopulations of 7,12-dimethylbenz[*a*]-anthracene-induced mammary tumors. Experientia **39**:283–285, 1983.
22. Brightwell J, Tseng MT. Peroxidase content in cell subpopulations of 7,12-dimethylbenz[*a*]anthracene-induced mammary tumors. Cancer Res **42**:4562–4566, 1982.
23. Tseng MT, Capuco AV. Estrogen receptor content in enzymatically dispersed, gradient enriched cell subpopulations of DMBA-induced mammary tumors in rat. IRCS Med Sci **9**:595–596, 1981.

Received April 18, 1983. P.S.E.B.M. 1983, Vol. 174.