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Response of 7,12-Dimethylbenz(a)anthracene-Induced Rat Mammary Tumor Cells to Tamoxifen, *in Vitro*[1] (41641)

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Abstract. Primary cell cultures from a density-defined cell subpopulation of the DMBA-induced rat mammary tumors were exposed to tamoxifen during their log phase of growth. Growth inhibition and the ultrastructure of surviving cells were examined along with the influence of this antiestrogen on the secreted proteins as determined by pulse labeling with [^{35}S]methionine and fluorography. Cell growth was remarkably inhibited at clinically achievable concentrations. However, ultrastructural changes in the surviving cells were minimal, the most noteworthy being the accumulation of myelin bodies. Protein secretion was affected in the defined subpopulation of several tumors by the reduced production of a high-molecular-weight protein. These tumors may represent a population of estrogen-sensitive tumors within the DMBA-induced mammary tumor model.

Many antiestrogenic compounds have been shown to inhibit the physiological influences of estrogen on target tissues and have been used successfully in treatment of advanced breast cancer in postmenopausal women (1–3). One such agent, tamoxifen (ICI 46474; *trans-p-β*-dimethyl-aminoethoxyphenyl-1-2-diphenyl-but-ene), has the ability to bind to the estrogen receptors of target tissues either competitively or allosterically (4, 5). Binding of tamoxifen (TAM) to intracellular estrogen receptors is the initial event leading to the binding of the tamoxifen-receptor complex to specific sites on nuclear chromatin, thus affecting DNA transcription and ultimately protein synthesis (6).

Recently, it has been reported that estrogen has the capability to induce the secretion of specific proteins in cultures of several human cell lines (7, 8). Westley and Rochefort (8) reported that two proteins, with molecular weights of 160,000 and 52,000 Da, were estrogen dependent and sensitive to tamoxifen. Additionally, these proteins were seen only in cell lines having high levels of estrogen receptors.

In this study, growth kinetics, ultrastructure, and protein secretion of surviving cells from cultures of a density-defined cell subpopulation of the 7,12-dimethylbenz(a)-anthracene-induced rat mammary tumor treated with tamoxifen were evaluated. With the results showing a remarkable cell-growth inhibition, accumulation of myelin bodies, and a reduction in a high-molecular-weight protein in several of the tumors tested.

Materials and Methods. *Cell separation and preparation of monolayer cultures.* Female Sprague–Dawley rats 50 days old were administered 7,12-dimethylbenz(a)anthracene (DMBA) by gastric intubation (9, 10). Approximately 6 weeks later 85% of the treated rats developed mammary tumors. Tumors were excised, trimmed of necrotic foci, sliced in 1-mm^3 blocks, and enzymatically disassociated (11). This cell suspension was layered upon a Ficoll (Sigma, St. Louis, Mo.) gradient (5–30%) and separated by isopycnic centrifugation into density-defined cell subpopulations, four to six cell bands being routinely observed (10). Monolayer cell cultures were prepared from the fourth band, due to their high concentration of epithelial cells and adequate cellular yield, by plating 8×10^5 cells per well in Falcon multiwell tissue-culture plates (16.4 mm diameter, Falcon Plastics, Oxnard, Calif.). Cultures were maintained in Medium-199 (M.A. Bioproducts, Washington, D.C.) supplemented with 10% fetal calf serum, hormones, and antibiotics (12). Cells were grown at 37°C in a high-humidity incubator with a 5% CO_2–95% air atmosphere.

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Cell kinetics. To define a growth inhibition curve, cells in the log phase of growth were treated with tamoxifen base (Stuart, Wilmington, Del.) at concentrations of 10^{-4}–10^{-9} *M*. Twelve hours later, the tamoxifen-containing medium was removed, the cultures were washed with M-199, and cells were harvested by use of trypsin-versene (M.A. Bioproducts, Washington, D.C.). Cells were counted on a Coulter counter (Coulter Electronics, Hialeah, Fla.) and the number of attached cells, after treatment with various concentrations of tamoxifen, was expressed as a percentage of the control. The viability of attached cells was checked by dye exclusion and all cultures had greater than a 95% viability. In addition, cultures after tamoxifen treatment were pulse labeled for 30 min with 1 μCi/ml [^{3}H]thymidine (Sp. Act. 6.7 Ci/m*M* New England Nuclear, Boston, Mass.). Cultures were then fixed in 10% neutral buffered formalin, dehydrated, and autoradiography was performed as previously reported (13).

Cell recovery was determined after 12 hr of continuous tamoxifen treatment. Twenty-four hours after plating, cells were either treated with 10^{-5} *M* TAM or 10% fetal-calf-serum-supplemented medium. After the treatment period, treated cultures were either maintained in 10^{-5} *M* TAM medium or were washed twice and replenished with supplemented medium. Sequential samples were taken over the next 4 days and cell counts were made.

Radiolabeling and scintillation counting. Three days after the initial plating, cells were washed with M-199 and labeled for 12 hr with 75 μCi of [^{35}S]methionine (1000 Ci/m*M*, NEN, Boston, Mass.). Cells were then washed twice and reincubated with fresh medium. A portion of the final wash was retained and used to measure the starting radioactivity (time zero). The medium was collected and replenished at hourly intervals during the next 6 hr. Cellular debris was removed by centrifugation and proteins were precipitated with trichloroacetic acid and added to the scintillation cocktail (Aqusol, NEN, Boston, Mass.) for determination of [^{35}S]methionine incorporation into proteins.

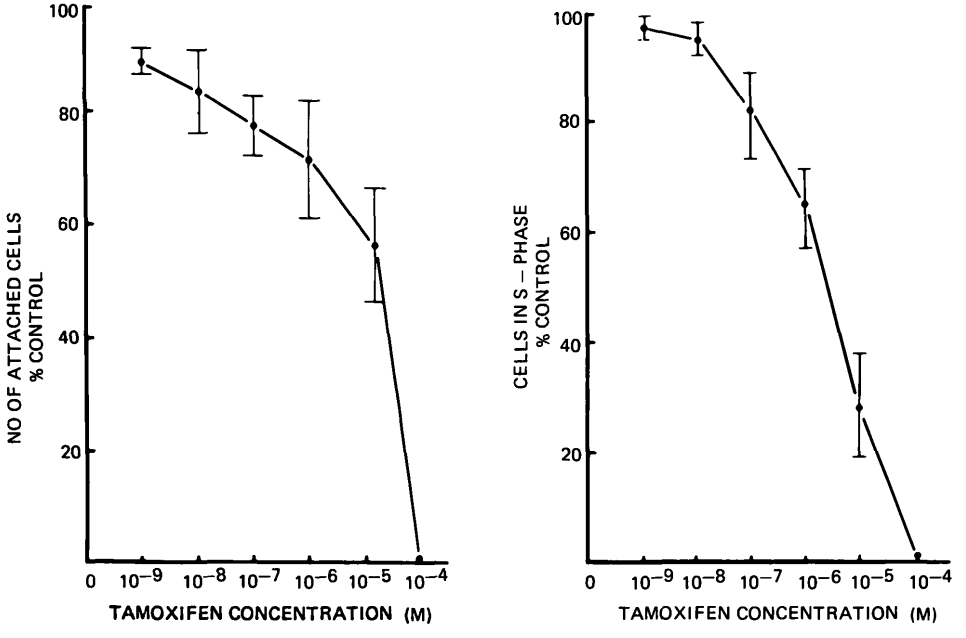

FIG. 1. Dose-response curve (left) of tamoxifen treatment of the DMBA-induced rat mammary tumors in primary cultures. Each point represents the average response of four tumors. Effects of tamoxifen (right) on [^{3}H]thymidine incorporation. Each point represents the average of four tumors obtained after counting a minimum of 1000 cells for each tumor.

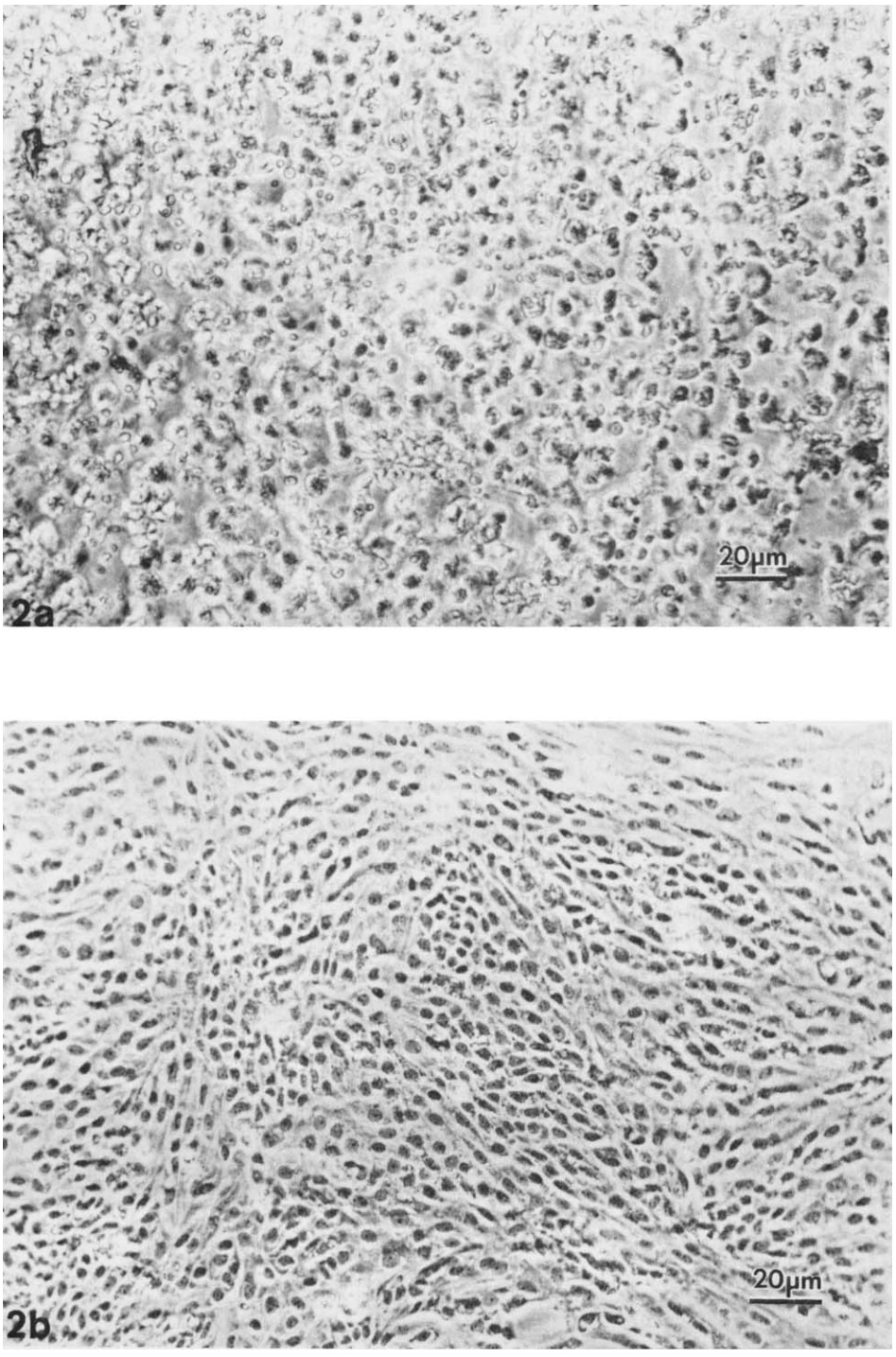

FIG. 2. (a) A primary culture of the DMBA-induced rat mammary tumor cell subpopulation after treatment with 10^{-4} *M* tamoxifen. Note the spherical shape of the cells and their detachment from the substrate. (b) A primary culture of the DMBA-induced cells after treatment with 10^{-9} *M* tamoxifen. Note the cobblestone appearance of the epithelial cells growing in monolayer culture.

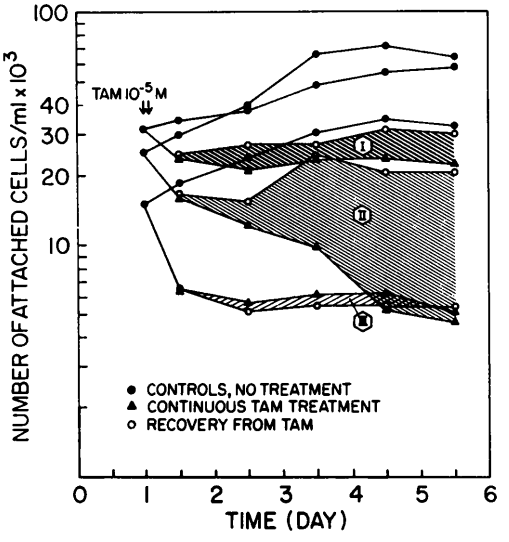

FIG. 3. Cultures from three DMBA-induced tumors that were either maintained continuously in tamoxifen (10^{-5} *M*) or replenished with supplemented medium, after which cell growth was followed for the next 4 days. Control cultures (●) show a continuous increase in cell number, whereas continuous drug treatment (O) shows either a continuous decline (II) or a plateauing (I and III) after the proportion of hormone-sensitive cell were destroyed. Cultures in which the drug-containing medium was removed after 12 hr and replenished with supplemented medium (▲) reveals that cell recovery was either rapid (II) or minimal (I and III) in response to the estrogen-containing fetal calf serum.

SDS gel electrophoresis and fluorography. Sodium dodecylsulfate (SDS) gel electrophoresis was performed by the addition of 50 μl of the protein-containing medium with an equal volume of sample buffer (14) and heating to 100°C for 2 min. Samples were analyzed on a 1.5-mm thick 7.5% acrylamide slab gel with a 3% stacking gel, both containing 1% SDS. Gels were run in a cold room (4°C) at 1.5 mA/sample overnight. Fluorography was performed according to methods described by NEN (Boston, Mass.). Gels were dried on a slab-gel dryer (Bio-Rad, Richmond, Calif.) and placed on Kodak X-omat AR film (Eastman Kodak Co., Rochester, N.Y.) at −80°C for approximately 6 days. Films were then developed according to standard Kodak developing procedures.

Electron microscopy. For ultrastructural analysis cells were taken after a 12-hr treatment period, fixed, dehydrated, and embedded in Araldite 502 as described earlier (12). Cells were then sectioned, stained with lead citrate and uranyl acetate, and examined on a Phillips 300 transmission electron microscope.

Results. At the lower tamoxifen concentrations (10^{-8}–10^{-9} *M*) 80–90% of cells survived 12 hr of treatment. In these cultures the proportion of cells entering the S-phase of cell division were nearly equal to those of the control (Fig. 1). This minimal change at low concentrations gradually yielded to both an increasing cell kill and inhibition of [^{3}H]thymidine incorporation at higher drug concentrations. At the highest concentration (10^{-4} *M*) TAM completely destroyed the cultures. Light microscopic examination revealed that cultures treated with TAM at 10^{-9} *M* retained their cobblestone appearance, whereas those treated with 10^{-4} *M* TAM rounded up and detached from the substrate (Fig. 2). Cell recovery after 12 hr of exposure to 10^{-5} *M* TAM, the LD_{50} dose, varied among cultures from different DMBA-induced tumors (Fig. 3). After 12 hr of treatment, the number of surviving cells is approximately 50% of the control cultures. With continuous TAM treatment some cultures showed a moderate reduction in cell number which was followed by a plateauing of the cell population (Fig. 3, I and III), while others exhibited a continuous decline in the number of cells surviving the treatment (Fig. 3, II). Conversely, with the addition of 10% fetal-calf-serum-supplemented medium, some tumors demonstrated a rapid regrowth (Fig. 3, II) while others exhibited little response (Fig. 3, I and III).

Ultrastructural examination of TAM-treated cultures revealed that myelin bodies accumulated in a dose-dependent fashion. These membranous whorls were not observed in control cultures (Fig. 4a) but gradually appeared as the concentration of TAM was elevated. In cultures exposed to 10^{-5} *M* TAM, nearly all cells contained one or more myelin bodies (Fig. 4b) and except for a slight increase in heterochromatin in the nuclei, no other obvious structural changes were observed. At 10^{-4} *M* TAM, only cellular debris and cells in the later stages of degeneration were seen, i.e., pycnotic nuclei with detaching

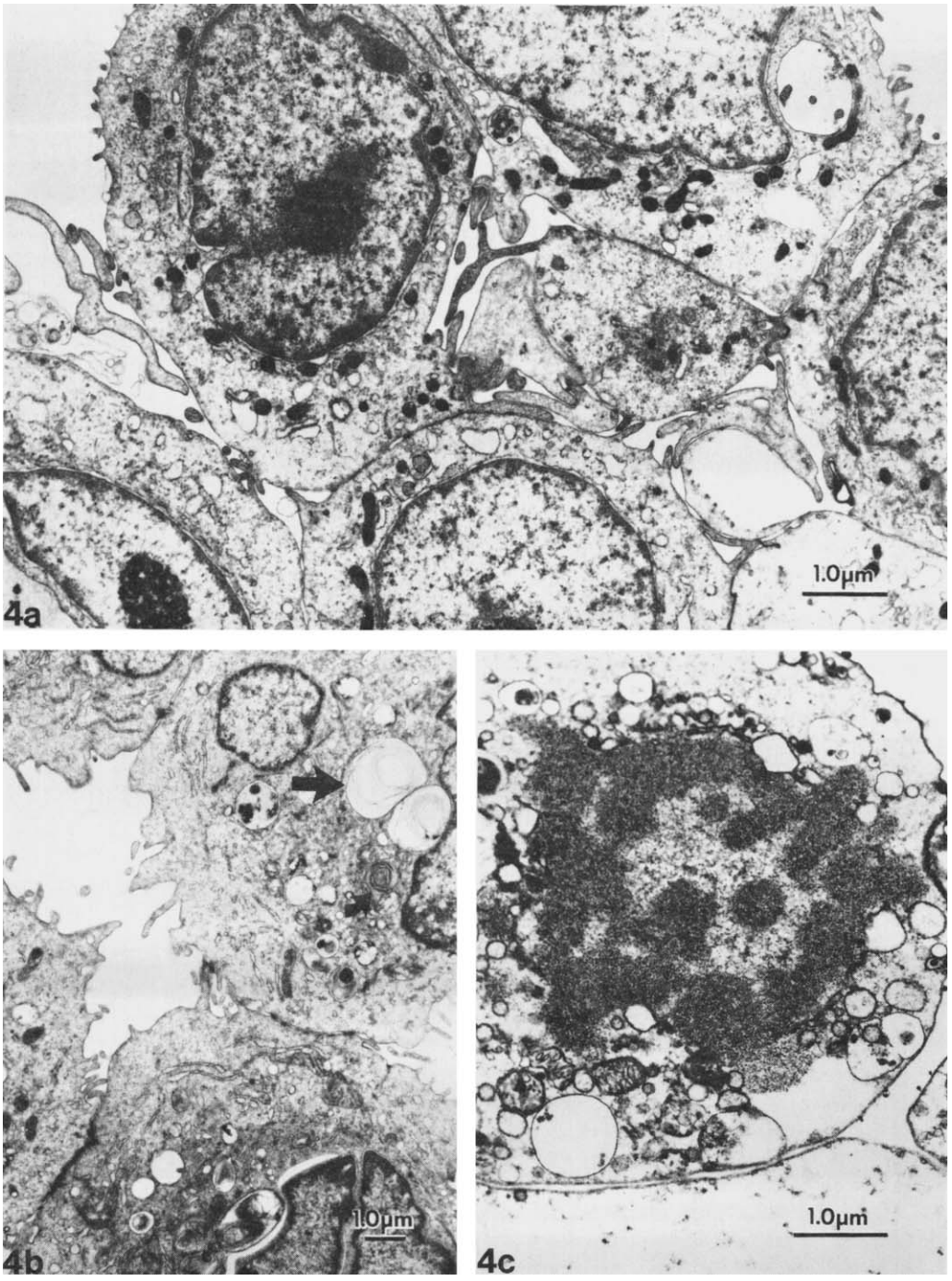

FIG. 4. (a) Cultured tumor cells before tamoxifen treatment. Note the absence of concentric membranous whorls. (b) Cultured tumor cells exposed to 10^{-5} *M* tamoxifen for 12 hr. Multiple myelin bodies (arrows) and clumping of the chromatin are evident. (c) Cultured tumor cells after 10^{-4} *M* tamoxifen treatment. Extensive chromatin clumping and detaching nuclear membrane can be observed in this degenerating cell.

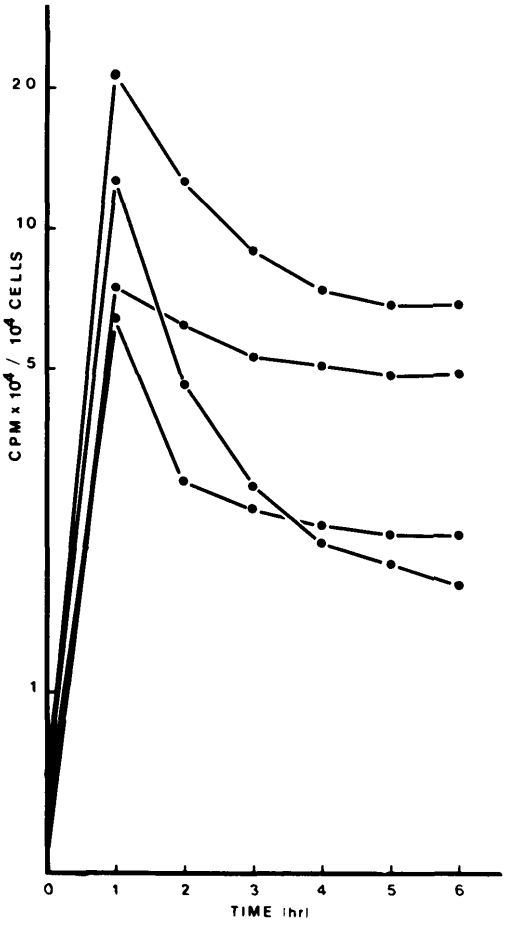

FIG. 5. Time course for secretion of proteins labeled with [^{35}S]methionine. Peak secretion levels of the labeled proteins occurred 1 hour after removal of the [^{35}S]methionine, this is followed by a rapid decline and plateauing for the next several hours. The graph shows the time course of secretion for each of four separate tumors.

nuclear membranes and perforated plasmalemma (Fig. 4c).

During a 12-hr period the incorporation of [^{35}S]methionine into the cells appeared to have been rapid. Labeled methionine was incorporated into secreted proteins with the peak in secretion occurring within 1 hr (Fig. 5). This was followed by a rapid decline in the secretion of the labeled protein which became plateaued within 3 hr. Electrophoretic analysis of the medium at the peak of secretion revealed two types of secretion profiles among the 10 primary cultures tested (Fig. 6). There is a difference in the proteins secreted by these two tumor types. Type II profiles showed a reduction in two proteins, one a 66,000-Da protein and the other an approximately 140,000-Da protein, that were apparent in type I cultures as dense bands. In response to tamoxifen, a future difference can be shown between the two profiles. Type I profiles exhibited a reduction in a 160,000-Da protein, whereas type II profiles show no differences between controls and treated.

Discussion. In this study we have demonstrated a marked decrease in both cell survival and [^{3}H]thymidine incorporation at TAM concentrations greater than 10^{-6} *M* (0.3 μg/ml). These results corroborate earlier reports indicating that plasma concentrations of TAM greater than a 0.15 μg/ml in patients often lead to a tumor regression (15). Additionally, studies in rats (16) reveal that oral administration of 20 mg/ml yields peak serum levels of TAM between 0.3–3.0 μg/ml (10^{-6}–10^{-5} *M*). These observations coupled with the changes observed in the cultures indicate that TAM exerts both cytotoxic and antimitotic effects in a dose-dependent fashion. Our cell recovery data (Fig. 3) illustrates a phenomenon common to primary culture systems, i.e., heterogeneity of the cell population within a tumor (17–19). This is manifested both by differences in the response to continuous drug treatment, regrowth of cells after drug removal, and the addition of medium containing physiological concentrations of estrogens. These differences in response may reflect the presence of hormone-dependent and hormone-independent cells within the tumors, or differences in their proportions. Although some tumors may exhibit a tumor cell population in which the majority of the cells are hormone dependent (Fig. 3, II), other tumors may have a larger proportion that are hormone independent (Fig. 3, I and III).

Ultrastructurally, the surviving cells showed accumulation of myelin bodies and an increase in heterochromatin at intermediate doses of TAM. At the highest dose (10^{-4} *M*), only degenerating cells and cellular debris were seen. Myelin body formation has been attributed to being modified secondary lysosomes or hypertrophied smooth endoplasmic retic-

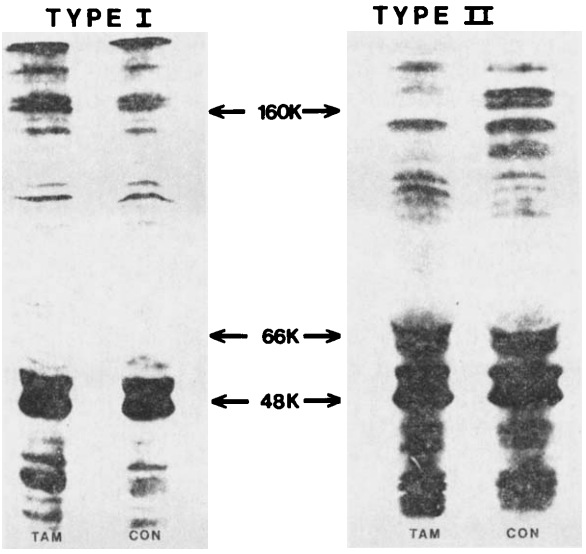

FIG. 6. SDS–polyacrylamide gels of secreted proteins. Two types of secretion profiles are shown; type I showing a change in a high-molecular-weight protein and type II showing no change in the secreted proteins. Control cultures (CON) are shown on the right and cultures representing 10^{-5} *M* tamoxifen treatment (TAM) are shown on the left. Type-I cultures were seen in 3 out of the 10 tumors tested by PAGE.

ulum (20, 21). They have been most often observed in hepatocytes but are not confined to these cells (21–23). Myelin bodies are induced by several drugs and are characterized by their concentric form (21, 24, 25). Myelin bodies induced by TAM are unicentric and have been described as local areas of cytoplasmic degradation in response to a pathological condition or drug treatment (21, 23). Increased heterochromatin may reflect the antimitotic effects of TAM and could represent the inactivation of DNA with transformation of the chromatin from the euchromatic to the inactive heterochromatic state (26, 27).

In this study tamoxifen treated cells demonstrated two protein secretion profiles (Fig. 5). One was unchanged from controls whereas the other exhibited a reduction in a 160,000-Da protein. This effect may reflect the presence of different proportions of hormone-dependent and hormone-independent cells in the parent mammary tumors. Lippman *et al.* (7) reported that estradiol was capable of inducing new proteins in the MCF-7 cell line. Later, Westley *et al.* characterized these newly synthesized proteins as being responsive to the effects of TAM and having molecular weights of 46,000, which was recently revised to 52,000 Da (22), and 160,000 Da (8). It is of interest to note that the 52,000-Da protein, if indeed present in the DMBA-induced mammary tumor model, was not sensitive to the effects of TAM. The identity of the 52,000-Da protein reported by Westley *et al.* (8) in several human cell lines remains unknown. Regardless of its identity, it has been shown to be an estrogen-dependent protein seen only in cell lines which are estrogen-receptor positive (8). In this regard it is interesting to note the absence of the 52,000-Da moiety in the cultured DMBA-induced mammary tumor cells which are also estrogen-receptor positive (10, 27). While Westley *et al.* (8) suggested that a 52,000-Da protein assay maybe of value in predicting a patient's response to hormonal therapy, our study points to the 160,000-Da protein as a better marker for estrogen-dependent tumors due to its more wide occurrence in estrogen-receptor positive and hormone-dependent tumors.

In summary, we have characterized the effects of the antiestrogen tamoxifen on a density-defined cell subpopulation of the DMBA-

induced rat mammary tumor in primary culture. These include the ability to induce both cytotoxic and antimitotic effects in the cultured cells, to affect changes in the production and secretion of the 160,000-Da protein, and to induce the formation of myelin bodies at higher concentrations.

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