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The Effects of 17β Estradiol and Tamoxifen on the ZR-75-1 Human Breast Cancer Cell Line in Defined Medium

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Absract—*The effects of 17β estradiol and tamoxifen on the ZR-75-1 human breast cancer cell line in serum free hormone supplemented medium are described. Addition of 17β estradiol leads to a marked stimulation of the breast cancer cells manifested by increases in cell number, precursor incorporation into macromolecules, liquid synthesis, and thymidine kinase activity. Concentrations of 17β estradiol as low as 10^{-11} M stimulate growth; 10^{-10}M 17β estradiol is maximally effective. Addition of the antiestrogen tamoxifen in cells devoid of estradiol leads to a period of estrogen reversible inhibition followed by cell death. 10^{-8}M tamoxifen has little effect on growth whereas 10^{-6}M tamoxifen is maximally effective. Hundred-fold lower concentrations of 17β estradiol when added simultaneously blocks antiestrogenic inhibition almost completely. These data obtained in a totally defined serum free system provide strong evidence for the absolute and direct growth dependence of some human breast cancer cells on estrogens.*

INTRODUCTION

IT HAS recently been shown that it is possible to grow cell lines in medium without serum supplementation provided the culture medium is supplemented with hormones and other factors [1, 2]. For example, Hayashi *et al.* [3] have shown that the GH_3 rat pituitary cell line will grow in Ham's nutrient mixture F-12 supplemented with L-triiodothyronine (T_3), thyrotropin releasing hormone, transferrin, parathyroid hormone, insulin, fibroblast growth factor and somatomedin C. This cell line and several other cell lines are able, even after long-term culture, to be adapted to serum-free growth [4, 5]. We have recently shown that the ZR-75-1 [6] human breast cancer cell line can be grown in hormone supplemented medium without serum [7]. The factors required for optimal growth (equivalent to that seen in serum supplemented medium) are 17β estradiol, insulin, transferrin, dexamethasone and T_3. Thus far most cells lines growing in a hormone supplemented medium have required insulin and transferrin although their effect on growth at identical concentrations varies among the cell lines. Also, each cell line has been suggested by Sato and colleague [3–5] to require a hormone which localizes in the nucleus such as T_3 or a steroid. The availability of a human breast cancer cell line which can be propagated in hormone supplemented medium without serum should aid in the study of the mechanisms by which hormones effect cell proliferation. We were particularly anxious to develop such a defined growth system in order to be able to study specific hormonal effects of estrogens and antiestrogens because of their central role in regulation of human breast cancer.

The requirement of virtually all established cell lines for serum has hampered the investigation of many aspects of hormone action. Serum contains growth factors, both known and undefined along with a wide variety of different hormones. These hormones and/or growth factors may cloud interpre-

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Abbreviations: L-Triiodothyronine, T_3; Eagle's minimal essential medium, MEM; Improved minimal essential medium, IMEM; Improved minimal essential medium—hormone supplemented, IMEM-HS; Charcoal treated calf serum, CCS; Phosphate buffered saline, PBS; 17β estradiol, E_2; Tamoxifen, T.

tation of hormone action data. Strobl *et al.* [8] have shown that MCF-7 cells, grown in fetal calf serum containing estradiol, retain estradiol for a prolonged period and furthermore that the estradiol is specifically bound and largely localized to the nuclear fraction.

Lippman *et al.* [9] have demonstrated stimulation of the MCF-7 cell line by estrogens and inhibition of growth of these cells by anti-estrogens. These experiments were performed with cells maintained and passaged in medium supplemented with charcoal treated calf serum (CCS) but the stimulation experiments were performed under serum free conditions. Although, the use of CCS insures a low concentration of estrogen, the serum-free conditions of these experiments do not support prolonged cell growth and both the controls and estrogen stimulated cells will eventually die despite obvious differences between estrogen treated and control cells.

Medium containing serum also leads to difficulties in evaluation of the hormone receptors of the cells. Two recent papers [10, 11] reported that, in MCF-7 cells grown in CCS, 75% of the total estrogen receptors exist in the unoccupied nuclear form. Cells grown in the presence of estradiol contained exclusively occupied nuclear receptors. It has also been recently shown that the progesterone receptor of MCF-7 cells is regulated by estradiol and cells grown in serum containing estrogens have higher concentrations of progesterone receptor than cells grown in CCS which contains very small amounts of estrogen [12].

In this report we examine the effects of 17β estradiol and the anti-estrogen tamoxifen on the ZR-75-1 human breast cancer cell line growing in a serum-free hormone supplemented medium. Their effects on cell growth, DNA synthesis, RNA synthesis, protein synthesis and lipid synthesis as assessed by thymidine incorporation, uridine incorporation, leucine incorporation and acetate incorporation into lipids respectively, are examined. Finally, we examine the effect of 17β estradiol on induction of the enzyme thymidine kinase.

MATERIALS AND METHODS

Cells

The ZR-75-1 cell line was used for all studies. This cell line has been described extensively in a previous publication [6]. Briefly, it is a human breast cancer cell line derived from a malignant ascitic effusion of a patient with infiltrating duct carcinoma of the breast. The cell line has been grown in Eagle's minimal essential medium (MEM) [13] supplemented with 5–10% fetal calf serum for greater than 3.5 yr and through 100 passages. These cells can also be propagated in a defined medium lacking serum improved minimal essential medium—hormone supplemented (IMEM-HS). This cell line has been shown to have specific receptors for estrogens, androgens, glucocorticoids and progestins, [6] as well as insulin [14].

Medium and hormones

Improved minimal essential medium (IMEM) [15] supplemented with L-glutamine (0.6 g/l), penicillin (62 mg/l) and streptomycin (135 mg/l) was the basic culture medium to which hormones and growth factors were added. All media was prepared in the NIH Media Unit. Transferrin (Sigma, St. Louis, Mo.) was added at a final concentration of 1 μg/ml (10^{-11}M). L-Triiodothyronine (Sigma, St. Louis, Mo.) 10^{-5}M stock solution was prepared in 0.1 N NaOH and added to medium to yield a final concentration of 10^{-8}M. Insulin U-100 (Eli Lilly and Co., Indianapolis, Ind.) was added at a concentration of 5×10^{-7}M. 17β Estradiol and dexamethasone (Sigma, St. Louis, Mo.) in benzene–ethanol were evaporated to dryness, dissolved in ethanol and stored at −20°C until use. Final concentrations in the medium were 10^{-8}M 17β estradiol and 10^{-8}M dexamethasone. The final concentration of ethanol is 0.1% and this concentration has no effect on the growth of the cells. Tamoxifen (ICI 46474) was similarly prepared.

Cell growth experiments

Cells growing exponentially in MEM + 5% fetal calf serum were suspended with trypsin–EDTA (trypsin 0.05%; EDTA 0.02%) and replicately plated in minimal essential medium supplemented with 5% charcoal treated calf serum [13]. The cells were plated into sterile six-well (35 mm) plastic tissue culture dishes (Linbro Scientific Inc., Hamden, Conn). After sufficient time for the cells to become adherent (usually 12–16 hr), the medium was changed to improved minimal essential medium supplemented with estradiol, L-triiodothyronine, insulin, dexamethasone and transferrin (IMEM-HS) or to IMEM-HS

minus 17β Estradiol [7]. After 24 hr the medium was replenished with fresh medium of identical composition. At various times, cells were collected by suspension in trypsin–EDTA and counted in a hemocytometer.

Precursor incorporation

In order to asses the effect of 17β estradiol and tamoxifen on thymidine incorporation, uridine incorporation and leucine incorporation in the ZR-75-1 breast cancer cells, radiolabelled [^{3}H] thymidine, [5$-^3$H] uridine or [^{3}H] leucine (Amersham–Searle) diluted in Dulbecco's phosphate-buffered saline (PBS) (0.20 g/l KCl; 0.20 g/l KH_2PO_4; 8.0 g/l NACl; 2.16 g/l Na_2HPO_4|.7 H_2O; pH 7.4) was added to each dish 1–2 hr before the cells were harvested. Each dish usually contained 1 μCi of radioactivity. Cells were harvested by washing the dishes once with PBS, suspended in EDTA (0.02%, and cell pellets collected by centrifugation. Cell pellets were suspended in water and sonically dispersed for 3 sec in a Branson sonicator (Branson Sonic Power Co., Danbury, Conn.) at the lowest setting. Aliquots were then used for the determination of protein using the method of Lowry [16] or thymidine, leucine or uridine incorporation by precipitation in 10% trichloracetic acid. Acid-insoluble counts were collected and washed on a 0.45 μm Millipore® filter. After drying, the filters were solubilized in Aquasol (New England Nuclear, Boston, Mass.) and counted in a Packard Scintillation Counter (Packard Instrument Co., Downers Grove, Ill.)

In order to assess the effect of 17 β estradiol on acetate incorporation into lipids, the cells were pulsed with [2$-^{14}$C] acetate. Each dish usually contained 1 μCi/ml of radioactivity. At the end of the labeling period the medium was removed and the cells rapidly washed once with PBS. Cells were then harvested in 1 ml of PBS containing 0.02% EDTA. Each dish was washed once with 1 ml of PBS, and the wash added to the harvested cell suspension. Washing and harvesting were carried out at room temperature. The cells were centrifuged at high speed in a Clay–Adams Serofuge, and the resulting pellet resuspended in 1 ml of PBS. Samples were taken for protein determinations as above and the remaining cells recentrifuged. The cell pellet was incubated in 0.1 ml of 30% potassium hydroxide in 50% methanol for 2 hr at 90°C. The incubation tubes were cooled, acidified with 0.1 ml of concentrated hydrochloric acid and extracted once with 1 ml of hexane. Extraction was accomplished by mixing each tube for 15 sec on a Vortex–Genie mixer (Scientific Industries, Inc.) When [^{3}H] palmitic acid was dissolved in potassium hydroxide, acidified and extracted with hexane, recoveries of the radioactivity were greater than 93%. Aliquots (0.5 ml) of the hexane layer were then transferred to glass scintillation vials; 8 ml of Aquasol (New England Nuclear) were added, and radioactivity measured in a liquid scintillation spectrometer (Packard model 3044).

Assay of thymidine kinase activity

Cell pellets were harvested as before and suspended in 1 ml of Tris–sucrose buffer (50 μM Tris, pH 7.6+250 μM sucrose) prior to disruption with 30 strokes of a tight fitting Dounce homogenizer. The homogenate was centrifuged at 104,000 **g** for 10 min and the resulting supernatant was assayed for thymidine kinase as described below in a method modified slightly from Breitman [17].

Aliquots (5$-$15 μl) of the 104,000 **g** containing 5–30 μg of protein, were incubated at 37°C in a solution containing [CH_3-^3H] thymidine (1.85–4.3 μM), 5mM ATP, 5 mM $MgCl_2$, and 100 mM potassium Hepes buffer, pH 7.4; final volume, 0.1 ml. Aliquots (22 μl) of the assay mixture were withdrawn at time intervals to 15 min and pipetted onto Whatman DE-81 filters. After washing sequentially in 1 μM ammonium fornate, ethanol and ether, the filters were counted in Econoflor (NEN). Assays are linear with time and added protein.

RESULTS

Figure 1 illustrates the effects of various concentrations of 17β estradiol added to cells growing in IMEM-HS minus 17β Estradiol. The cells were plated in MEM plus 5% CCS and this medium was exchanged daily for 3 days. The medium was then changed to IMEM-HS minus estradiol and this medium was exchanged daily for 8 days. This long pretreatment of the cells with charcoal treated calf serum and estradiol free medium is required to completely remove estradiol in these cells. We have previously shown in cells treated with tritiated estradiol in which the medium was exchanged daily for fresh serum free medium lacking estradiol that 10–14 days are required for a 2 log reduction in retained

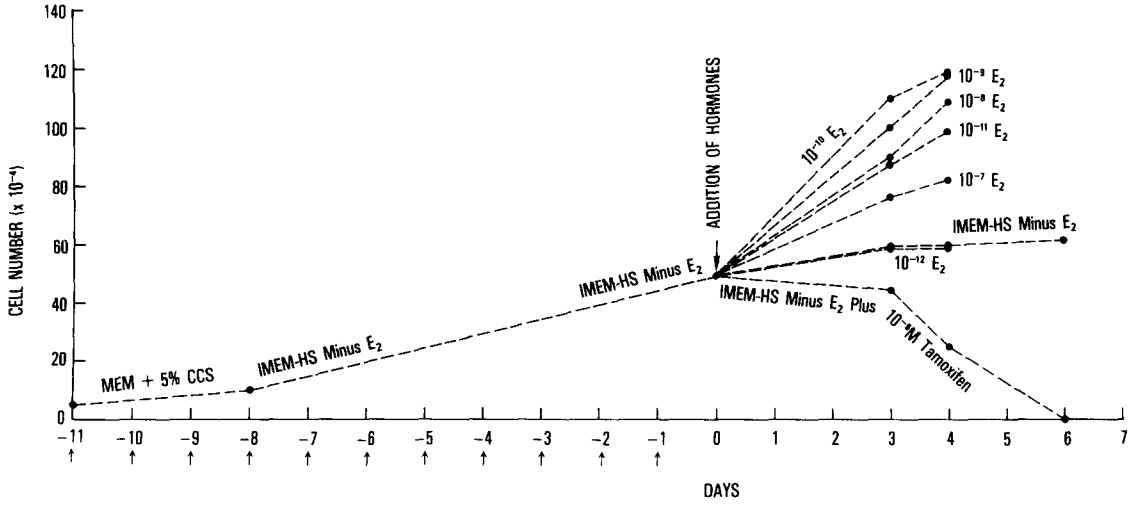

Fig. 1. The effect of 17β estradiol and tamoxifen on the ZR-75-1 human breast cancer cells. Cells were plated in MEM supplemented with 5% CCS. This medium was exchanged daily for 3 days. On day −8, the medium was changed to IMEM-HS minus E_2 and this medium was exchanged daily for 8 days. On day 0, various concentrations of estradiol and 10^{-6}M tamoxifen were added to the cells. Arrows indicate days on which the cells were refed with fresh medium. Standard deviations of triplicate cell counts shown are unusually less than 10%.

radioactivity [18]. In experiments on another cell line MCF-7, we have demonstrated that these retained counts are virtually all 17β estradiol, are specifically bound, and are largely localized to the nuclear fraction [8]. In addition, either charcoal treated serum, bovine serum albumin, antiestrogen or unlabelled estradiol addition can significantly accelerate loss of non-specifically and specifically retained estradiol. On day 0 various concentrations of 17β estradiol (10^{-7}–10^{-12}M E_2) or 10^{-6}M tamoxifen were added. Initially, the cells in IMEM-HS minus estradiol grow slowly and eventually they cease growth. It is important to note that while there is no net change in cell number in cells deprived of estradiol, this does not appear to be due simply to a cessation of growth for the following reasons: First, estradiol free cells continue to incorporate thymidine though at a

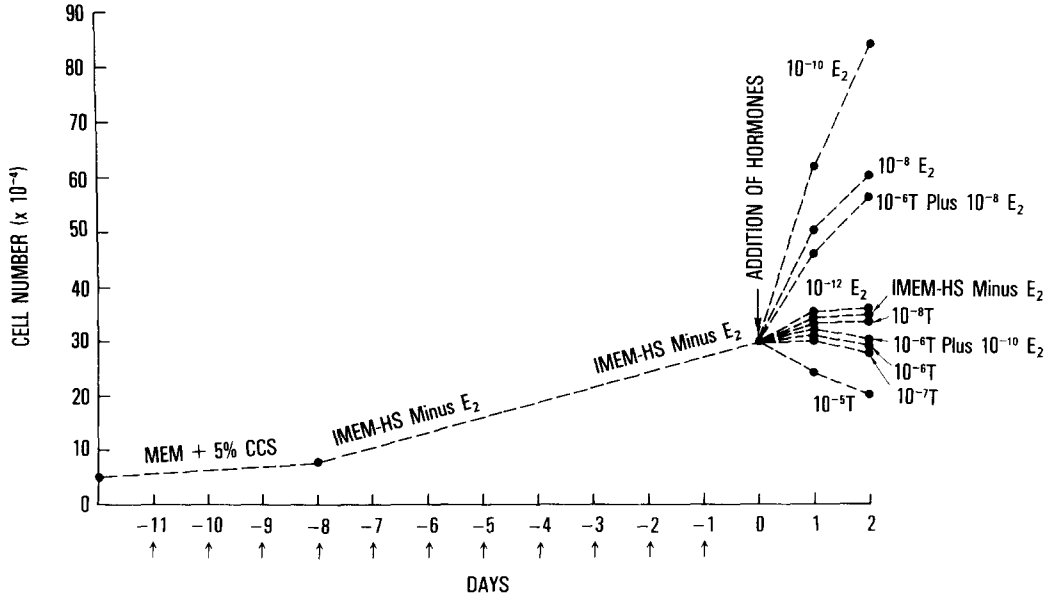

Fig. 2. The effect of 17β estradiol and tamoxifen on the ZR-75-1 human breast cancer cells. Cells were plated in MEM supplemented with 5% CCS. This medium was exchanged daily for 3 days. On day −8. the medium was changed to IMEM-HS minus E_2 and this medium was exchanged daily for 8 days. On day 0, various concentrations of estradiol, tamoxifen and estradiol plus tamoxifen were added to the cells. Arrows indicate days on which the cells were refed with fresh medium. Standard deviations of triplicate cell counts shown are usually less than 10%.

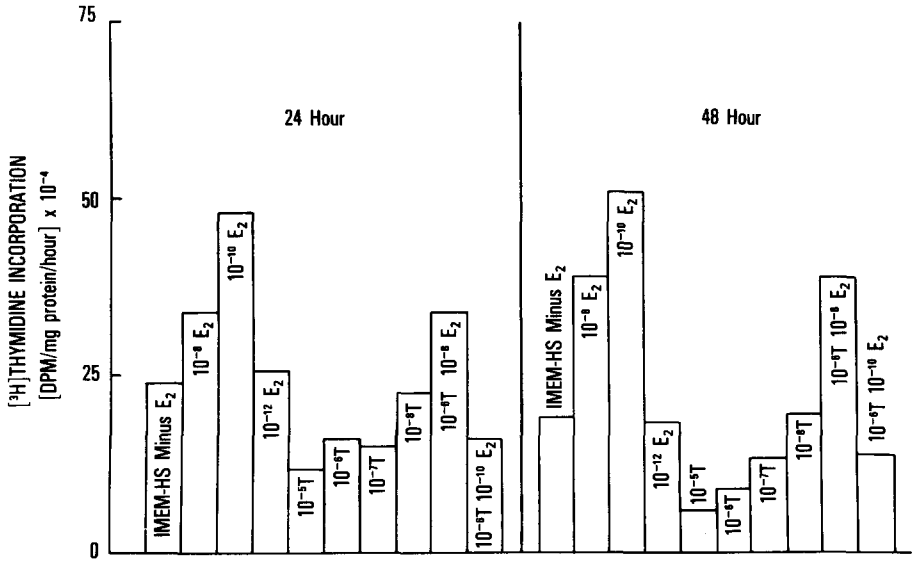

Fig. 3. The effect of 17β estradiol and tamoxifen on thymidine incorporation. Cells were maintained in IMEM-HS minus E_2 for 14 days with daily exchanges for fresh medium. At time 0, various concentrations of estradiol, tamoxifen and estradiol plus tamoxifen were added to the cells. Thymidine incorporation was measured at 24 hr and 48 hr. The height of the bars equals the mean thymidine incorporation (triplicate determinations). Standard deviations of the mean are usually less than 5%.

lower rate than hormone treated cells (see Fig. 3); second, if the cells are prelabelled with thymidine there is a loss of radioactivity into the medium in estrogen deprived cells but not in estrogen treated cells (estrogen treated cells, 1% loss; estrogen deprived cells, 60% loss); third, there is an obvious decrease in cell adhesiveness in estrogen deprived cells and detached cells are easily seen in the medium. Thus, it is likely that the ZR-75-1 cells are capable of low growth in estrogen free medium, an effect masked by continued cell loss from the dish and replenishment. Addition of 10^{-7}–10^{-11}M estradiol restores growth and adhesiveness to these cells. 10^{-9}–10^{-10}M appear to be the optimal concentration. No effect is seen with 10^{-12} E_2. Addition of 10^{-6}M tamoxifen leads to cell death.

Figure 2 shows a second growth experiment in which the interaction of estrogen and antiestrogen is examined in more detail. Again, maximal effects on increasing cell number were observed at 10^{-10}M E_2 and no effect was observed at 10^{-12} M. Again 10^{-6} M tamoxifen added to cells devoid of estradiol led to cell death. This was also observed at 10^{-5}M tamoxifen and 10^{-7}M tamoxifen but there was no effect at 10^{-8}M tamoxifen. Similar results were also observed with nafoxidine (data not shown). The cytotoxic effect of 10^{-6}M tamoxifen was nearly totally blocked by simultaneous addition of 10^{-8}M estradiol but not by addition of 10^{-10}M E_2.

In Figure 3 the effects of various concentrations of 17β estradiol and tamoxifen on thymidine incorporation are examined. Both the hormone and antihormone were added to cells which were in IMEM-HS minus E_2 for 14 days with daily media changes. After either 24 or 48 hr, addition of estradiol leads to an increase in thymidine incorporation over controls whereas addition of tamoxifen leads to a marked decrease in thymidine incorporation. Again, as in the growth experiments, the simultaneous addition of 10^{-8}M E_2 with 10^{-6} M tamoxifen is able to block the tamoxifen effect.

The time course of tamoxifen reversal by 17β estradiol is shown in Figure 4. The cells were maintained in IMEM-HS minus E_2 for 14 days. At time 0, 10^{-6}M tamoxifen was added to the cells. At times 0, 12, 24, 36, 48 and 60 hr, following the addition of tamoxifen, 10^{-9} M estradiol was added to the cells followed by measurement of thymidine incorporation 24 hr later for 1 hr. Addition of 10^{-9}M 17β estradiol between 0 and 36 hr after addition of 10^{-6}M tamoxifen leads to a reversal of the tamoxifen effect as measured by thymidine incorporation. No reversal is observed when 17β estradiol is added later than 60 hr, and in experiments not shown, the addition of 10^{-9}M E_2 72 hr after the addition

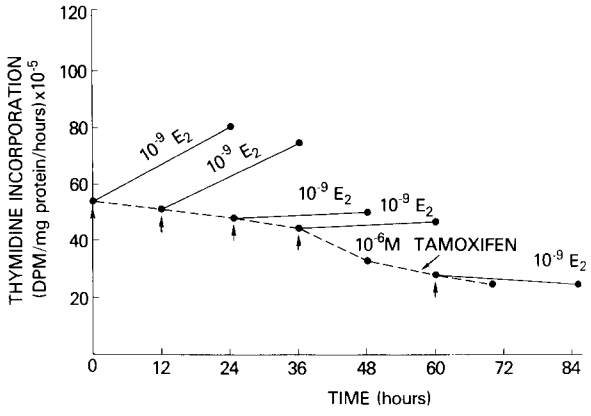

Fig. 4. The time course of the estradiol reversal of the cytotoxic effect of tamoxifen. Cells were maintained in IMEM-HS minus E_2 for 14 days. *At time* 0, 10^{-6}M *tamoxifen was added to the cells. At times* 0, 12, 24, 36, 48 *and* 60 hr, *following the addition of tamoxifen,* 10^{-9} M *estradiol was added to the cells followed by measurement of thymidine incorporation* 24 hr *later. Data points represent means of triplicate determinations. Standard deviations of the means are usually less than* 5%.

of 10^{-6}M tamoxifen still results in cell death.

Figure 5 illustrates the time course of 10^{-9}M E_2 on thymidine incorporation. The addition of estradiol to cells maintained in IMEM-HS minus E_2 for 14 days leads to a marked increase in thymidine incorporation as compared to controls. This effect is seen at 12 hr though not at 6 hr and is maximal between 24 and 36 hr. The effect on thymidine incorporation remains at about 50% above control levels between 48 hr and 72 hr. The data are expressed as both DPM/mg protein/hr and DPM/dish/hr. The percentage increase above control appears less in the data expressed as DPM/mg protein/hr because of the increasing amount of protein/dish which is secondary to cell growth. Note that the protein maximally at about 24 hr and remains constant as the cells have reached approximate confluency. The apparent decrease in thymidine incorporation after maximal effects have been attained is probably due to an effect of increased cell density. This phenomena is also observed in Figs. 6, 7 and 8 which examine the effect of estradiol on [5 $-^3$H] uridine incorporation, [^{14}C] leucine incorporation and [^{14}C] acetate incorporation. In these three experiments, as with the experiment measuring thymidine incorporation, 10^{-9}M E_2 was added to the cells maintained for 14 days in IMEM-HS minus E_2. The time course of estradiol stimulation for uridine incorporation and acetate incorporation are similar to that observed and described for thymidine incorporation. With respect to protein synthesis, however, (Fig. 7) the effects of estradiol are seen earlier (6 hr), although the maximal effect is still observed between 24 and 36 hr. Figure 9 shows the effect of addition of 10^{-9}M E_2 to cells growing in nucleoside free IMEM-HS minus E_2 on thymidine kinase activity. The peak effect of estradiol on thymidine kinase activity occurs 24 hr after E_2 addition.

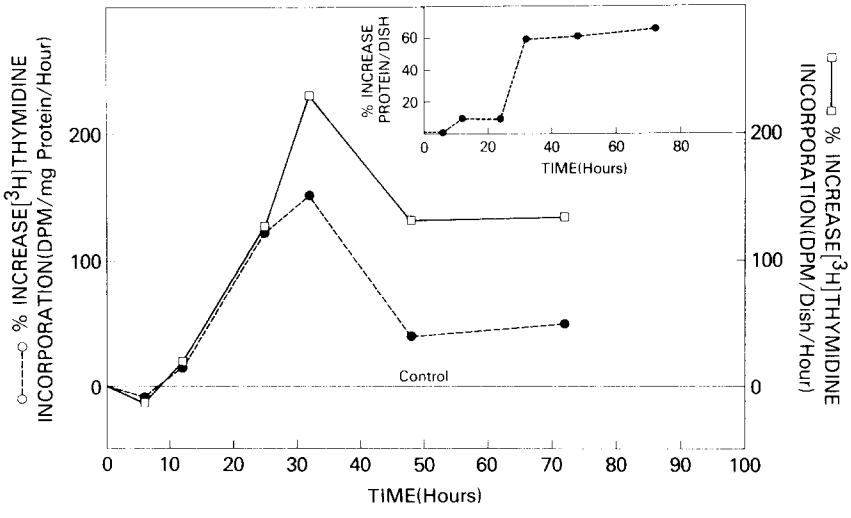

Fig. 5. The effect of 17β *estradiol on thymidine incorporation and cell growth. Cells were maintained in IMEM-HS minus E_2 for* 14 days *with daily medium changes. At time* 0, 10^{-9}M E_2 *was added to the cells. Data points represent the means of triplicate determinations. Standard deviations of the means are usually less than* 5%. *Data are expressed as* dpm/mg protein/hr ●---●, *or* dpm/dish/hr □—□. *Cell growth is expressed as percentage increase* protein/dish.

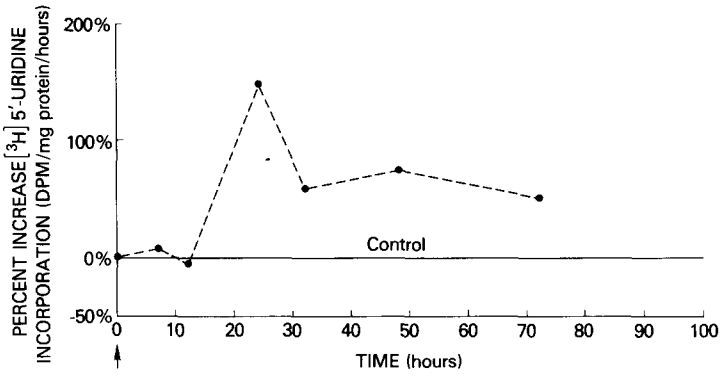

Fig. 6. Incorporation of [5-^{3}H-] *uridine by the ZR-75-1 human breast cancer cells after estradiol addition. Cells were maintained in IMEM-HS minus* E_2 *for* 14 days *with daily medium changes. At time* 0, 10^{-9}M E_2 *was added to the cells. Data points represent the means of triplicate determinations. Standard deviations of the means are usually less than* 5%.

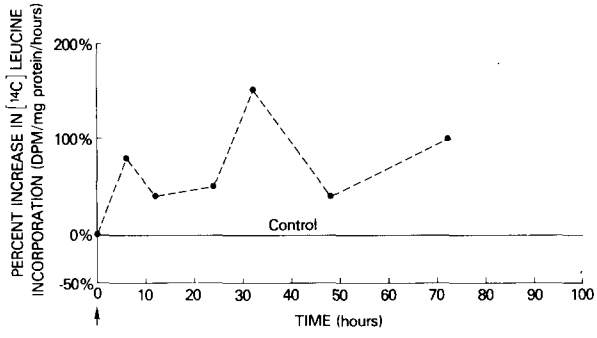

Fig. 7. Incorporation of [^{14}C] *leucine by the ZR-75-1 human breast cancer cells after estradiol addition. Cells were maintained in IMEM-HS minus* E_2 *for* 14 days *with daily medium changes. At time* 0, 10^{-9}M E_2 *was added to the cells. Data points represent the means of triplicate determinations. Standard deviations of the means are usually less than* 5%.

DISCUSSION

Estrogens have been shown to stimulate the proliferation of target tissues but the mechanism of this stimulation is still incompletely understood. Much of the difficulty in understanding this mechanism is due to the fact that previous *in vitro* studies have been performed on mixed and/or dying cell populations. These cells have often been in medium supplemented with serum containing physiologic levels of many hormones and also other defined and undefined serum factors.

Lippman *et al.* [9] have demonstrated stimulation of the MCF-7 human breast cancer cell line by estrogens and inhibition of growth of these cells by anti-estrogens. These earlier experiments were performed under serum free conditions with cells maintained and passaged in medium supplemented with CCS. Although the use of CCS insures a low concentration of estrogen, the conditions of these experiments are incompatible with prolonged survival of the cells in culture and both the controls and estrogen stimulated cells will eventually die. Furthermore, in these earlier experiments [9], the tamoxifen inhibition of the MCF-7 cells below control levels is difficult to interpret. If tamoxifen acted solely by

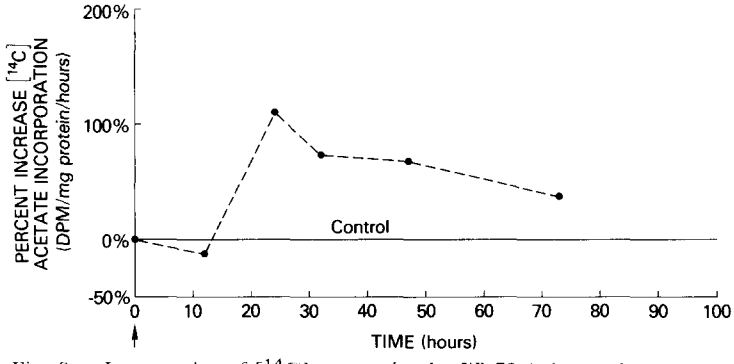

Fig. 8. Incorporation of [^{14}C] *acetate by the ZR-75-1 human breast cancer cells after estradiol addition. Cells were maintained in IMEM-HS minus* E_2 *for* 14 days *with daily medium changes. At time* 0, 10^{-9}M E_2 *was added to the cells. Data points represent the means of triplicate determinations. Standard deviations of the means are usually less than* 5%.

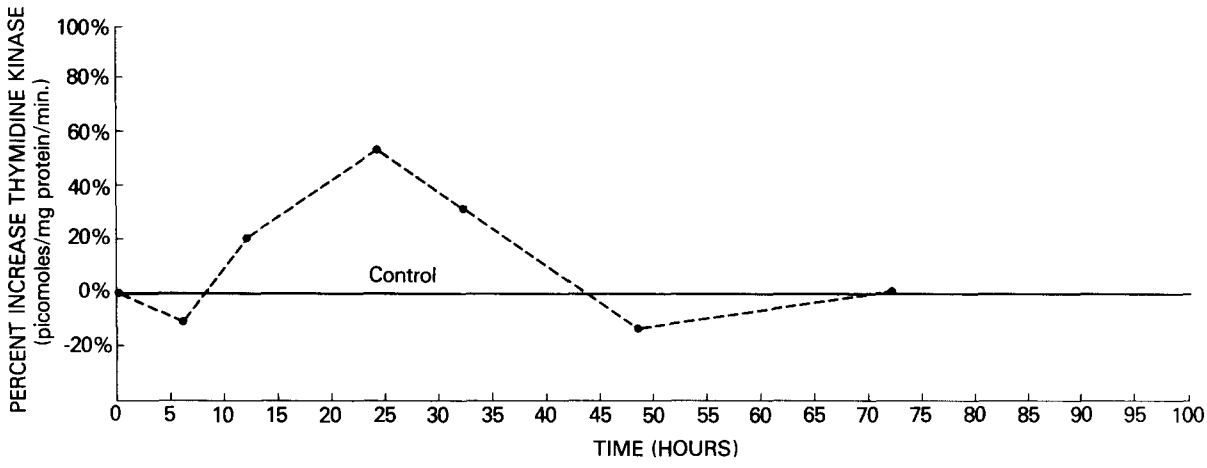

Fig. 9. Thymidine kinase activity in the ZR-75-1 human breast cancer cells after estradiol addition. Cells were maintained in IMEM-HS minus E_2 for 14 days *with daily exchanges for fresh medium. At time* 0, 10^{-9}M E_2 *was added to the cells. Data points represent the means of duplicate determinations. Standard deviations of the mean are usually less than* 10%.

competing with available estradiol for receptor then one would not expect inhibition of cell growth below control levels. It is possible that tamoxifen also has a specific effect on the cells mediated by anti-estrogen binding to the estrogen receptor and such a problem can be more readily explored in a defined medium.

In this study, we have presented data characterizing the response of the ZR-75-1 human breast cancer cell line in continuous culture to estrogen and anti-estrogen treatment. Several features of this system are noteworthy.

First, all of the experiments were performed with ZR-75-1 cells. This cell line has been carried in continuous culture for greater than 3 yr and through 100 passages. There is strong biochemical, morphologic and chromosomal evidence of its human breast cancer origin [6]. Second, all of the experiments were performed in totally defined, serum free, hormone supplemented medium. Using this medium which we have previously described [7], the effects of both 17β estradiol and tamoxifen were observed in an environment totally devoid of estrogen and in a system where although cells are serum free, are not dying. The cells are not committed to death, and will grow to confluence if fully supplemented with hormone. Third, in this defined environment (IMEM-HS minus 17β estradiol) we were able to show stimulation of the ZR-75-1 cells with estradiol at physiologic concentrations. Marked differences in cell number were observed as a function of estradiol concentration. 10^{-10}M E_2 produced maximal effects but increases in cell number were observed at a concentration range of 10^{-7}–10^{-11}M E_2. Furthermore, in cells devoid of estradiol for 14 days, the readdition of 10^{-9}M E_2 leads to marked increases in thymidine, uridine, leucine and acetate incorporation. Finally, in this defined environment, without estradiol, a marked but specific cytotoxic effect of tamoxifen was observed. This effect was dose dependent and reversible by estradiol. The mechanism for this cytotoxicity is still unknown; however, it can be prevented by the addition of estradiol simultaneously with the antiestrogen or reversed by estrogen addition within 60 hr of antiestrogen addition.

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