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MODEL SYSTEMS FOR THE STUDY OF ESTROGEN ACTION IN TISSUE CULTURE

MARC LIPPMAN,* GAIL BOLAN,* MARIE MONACO,* LAWRENCE PINKUS,† and LINDA ENGEL§

*Medicine Branch, National Cancer Institute;
†National Institute of Arthritis Metabolism and Digestive Diseases;
§Laboratory of Pathology, National Cancer Institute;
Bethesda, MD 20014 U.S.A.

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SUMMARY

Physiologic concentrations of estrogen stimulate precursor incorporation and growth in several lines of human breast cancer cells in long term tissue culture. Antiestrogens inhibit precursor incorporation and eventually kill the cells. Estrogens reverse antiestrogen effects. Responsive cell lines contain high affinity specific cytoplasmic receptors for estrogen. When binding and stimulation are measured under similar conditions, it appears that only a small proportion of receptor sites need be occupied for maximal stimulation. Thymidine kinase specific activity is increased by estradiol and may, in part, explain enhanced thymidine incorporation. These cell lines should prove useful for the study of the mechanisms by which estrogens regulate growth in human breast cancer.

INTRODUCTION

The fact that some human breast cancers may be estrogen dependent has long been appreciated. A variety of hormonal manipulations such as castration, and adrenalectomy as well as the administration of antiestrogens and large concentrations of estrogens have all been associated with clinical remissions in some patients [1]. The mechanisms by which these changes in the hormonal milieu influence the rate of growth of breast cancer have been widely investigated in clinical settings [2], organ culture [3–5] and animal models [6, 7]. Unfortunately these are complex systems in which the effects of one hormone on the levels or activity of others are difficult to unravel. In addition, in such systems, it may be difficult to separate out effects of a given hormonal manipulation on breast cancer cells themselves from those effects which may be mediated by an interaction with other tissues such as supporting stroma or the immune system. For these reasons we sought to establish and/or characterize the estrogen responsiveness of human breast cancer cell lines in tissue culture. The availability of such systems might permit a more exacting dissection of the pathways involved in estrogen dependence of breast cancer. In this report we review some of our recent work further characterizing the estrogen responsiveness of several cell lines in long term tissue culture.

METHODS

Cell lines

MCF-7 is a breast cancer cell line established from a malignant pleural effusion in a postmenopausal female with metastatic breast cancer [8] and generously provided by Marvin Rich of the Michigan Cancer Foundation. ZR75-1 was established by Nathaniel Young and Linda Engel of the National Cancer Institute. We have reviewed elsewhere [9–11] the criteria for the human and mammary nature of the MCF-7 cell line. The ZR75-1 has epithelial morphology by both light and electron microscopy and synthesizes α-lactalbumin (Young *et al.*: manuscript in preparation). Culture conditions are described elsewhere [9].

Binding assay

Our methods for cytoplasmic receptor assays and whole cell binding studies have been described previously [10]. We employ a competitive protein binding assay using dextran-coated charcoal to separate bound from unbound steroid cytoplasmic assays. Whole cell assays are done similarly except that following incubation with varying concentrations of radioactive and unlabelled steroids, cell bound and free steroid are separated by 3 washes in isotonic buffer.

Precursor incorporation experiments

Incorporation of radioactive nucleosides and amino acids into macromolecules were performed as outlined previously [9, 10] with one major exception. All of the experiments outlined in the present study were performed under entirely serum free conditions. 24 h prior to the start of an experiment, replicately plated cells growing in Improved Minimal Essential Medium supplemented with glutamine 0.6 g/L and antibiotics (IMEM) plus 10% mycoplasma free calf serum (North American Biological) were changed to IMEM without serum. 6 h later the medium was removed and fresh IMEM added. 18 h later various hormones were added as noted in the figure legends.

SDS polyacrylamide gels

Cells growing in IMEM plus 10% fetal calf serum were switched to serum free medium as before. After 24 h the medium was removed and fresh serum free IMEM was added. Beginning 24 h later 17β-estradiol (10^{-8} M) was added at various times to each flask such that all could be harvested together after respectively 0, 2, 5, 14, and 24 h of hormonal stimulation. Cells were homogenized in Tris–glycine buffer as described below and cytoplasmic extracts prepared by centrifugation at 104,000 *g* for 60 min at 4°C. Gel electrophoresis was carried out in 7.5% polyacrylamide gels containing 0.1% SDS in Tris–glycine buffer pH 8.6 and run as described elsewhere [12].

Assay of thymidine kinase activity

Cell pellets were harvested as before [10] and suspended in 1 ml of TS buffer (50 mM Tris, pH 7.6 + 250 mM 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 activity as described below [13]. Protein was determined by the method of Lowry[14]. In certain experiments cells were quick frozen in liquid nitrogen and stored at −70°C until use. This resulted in the loss of less than 10% of total thymidine kinase activity.

Aliquots (5–15 μl) of the supernatant containing 5–30 μg of protein, were incubated at 37° in a solution containing [CH_3-^{3}H] thymidine (2–4 μM), 5 mM ATP, 5 mM $MgCl_2$, and 100 mM Potassium HEPES buffer, pH 7.4; final vol. 0.1 ml. Aliquots (22 μl) of the assay mixture were withdrawn at time intervals to 15 s and pipetted onto Whatman DE-81 filters. After washing sequentially with 1 mM ammonium formate, ethanol, and ether, the filters were counted in Econoflor (New England Nuclear).

RESULTS AND DISCUSSION

The effects of 17β-estradiol and the antiestrogen Tamoxifen (ICI 46474) on incorporation of [^{3}H]-thymidine into acid insoluble material in the MCF-7 cell line are shown in Fig. 1. Forty eight h of estrogen treatment enhances thymidine incorporation about 2 fold, in the experiment shown. At the same time Tamoxifen inhibits thymidine incorporation to about 25% of control and 10% of estrogen stimulated values. If the cells are incubated in 10^{-7} M Tamoxifen plus 10^{-8} M estradiol, inhibition by the antiestrogen is partially overcome and thymidine incorporation is 2/3 that seen in cells stimulated with estradiol alone. Usually, reversal of antiestrogen is nearly complete and as little as 100 fold less 17β-estradiol is sufficient to overcome antiestrogen effects.

Similar effects of estrogen on thymidine incorporation are shown for the ZR75-1 in Table 1. Once

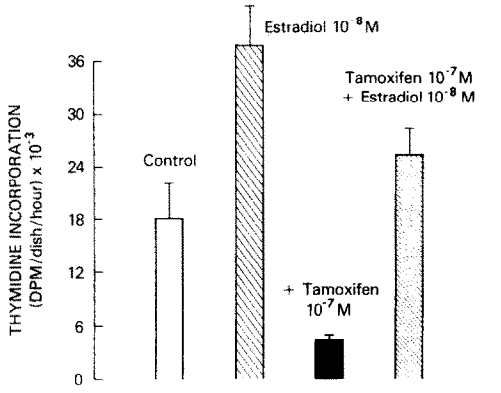

Fig. 1. Effects of 17β-estradiol and Tamoxiden (ICI 46474) on thymidine incorporation in MCF-7 human breast cancer. Following replicate plating and incubation of cells in serum free medium as described in Methods, hormones were added as 1000 × concentrates in ethanol. After 48 h in hormone, cells were pulsed with nucleoside for 1 h, harvested and acid insoluble radioactivity assessed as previously described [9]. Results are means of quadruplicate determination. ± 1 Standard deviation.

again estrogen stimulates and antiestrogen inhibits thymidine incorporation. The specificity of the inhibition by antiestrogen is suggested by its reversal by estrogen. In addition, as we have previously shown, antiestrogen effects once established can be reversed by subsequent estrogen administration, and Tamoxifen does not inhibit cell lines which lack estrogen receptor [9, 10]. Similar effects are seen when incorporation of RNA or protein precursors are measured. Furthermore, these increases in precursor incorporation are accompanied by an enhancement of the rate of cell division which is seen even under serum free conditions [10].

Much work has suggested that most if not all effects of steroid hormones are mediated by an initial interaction with cytoplasmic receptor [15]. The MCF-7 cell line has been previously reported to contain estrogen receptors [16] a result which we have confirmed [9, 10]. In Figs. 2 and 3 we contrast the specificity of binding of estrogen receptors in the established breast cancer cell line MCF-7 in tissue culture with the receptor in a tumor homogenate from a 62 year old female patient with breast cancer. The specificities of both these receptors are virtually identical. Material from this patient was used to start the ZR75-1 cell line. In both cases 17β-estradiol and the non steroidal estrogen, diethylstilbestrol, compete well with [^{3}H]-estradiol for specific binding sites. Antiestrogens also compete to the same extent but with a far lower apparent affinity. Other steroids such as androgens and progesterone show little ability to compete with estradiol for binding. Thus not only do these tissue culture cells appear to be responsive to estrogen but the receptors found in them appear to resemble at least by specificity studies the receptors found in fresh malignant material.

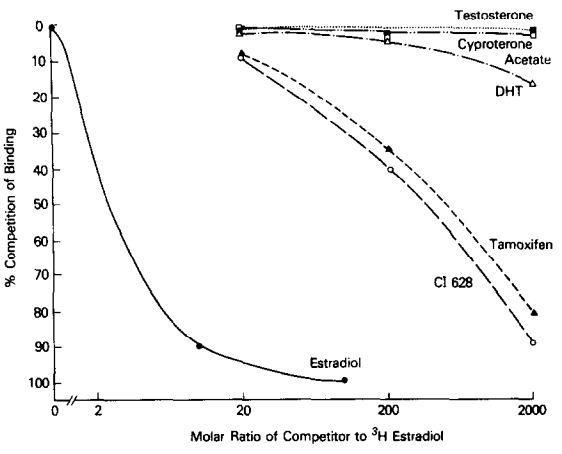

Fig. 2. Binding specificity of the estrogen receptor in MCF-7 human breast cancer. Following a 24 h incubation in serum free IMEM, MCF-7 cells were harvested in trypsin-EDTA and cytoplasmic extracts prepared [9]. These were incubated overnight at 4°C with 5×10^{-9} M [^{3}H]-17β-estradiol (100 Ci/mmol Amersham) with or without unlabelled steroids as shown in the figure legends. At the end of the incubation period bound and free steroid were separated with Dextran-coated charcoal.

Furthermore, as shown in Fig. 4 there is reasonable agreement between concentrations of estrogen which specifically bind to receptor sites in intact cells at 37°C, and concentrations of estrogen which stimulate thymidine incorporation. It is important to recall that binding and stimulation as shown in Fig. 4 are performed under essentially identical conditions with respect to temperature and media. As shown, the dose response curve lies to the left of the binding curve. This suggests that not every cytoplasmic receptor site need be occupied for maximal hormonal stimulation. This figure also emphasizes the sensitivity of these cells to estradiol. As little as 2–3 $\times$ 10^{-11} M 17β-estradiol reproducibly stimulates thymidine incorporation above control levels. The K_D of the receptor (in intact cells at 37°C) is 5.2×10^{-10} M ($r = 0.982$) and the intercept of the Scatchard plot shown in the inset is equivalent to 15,200 receptor sites per cell.

The data shown in Figs. 2 and 3 suggest that antiestrogens have a much lower affinity for estrogen receptor than 17β-estradiol. If antiestrogen effects also involve an interaction with receptor, then one might predict that antiestrogen effects would require higher concentrations than estradiol. This result is observed in Fig. 5. Though, as shown in the figure, there is relatively good agreement between concentrations of antiestrogen which displace estradiol from receptor, this does not mean that this is the way in which they exert their effects. It should be noted that as shown in Fig. 1, and in our previous work [9, 10], antiestrogen alone is more inhibitory than control medium lacking hormone. This suggests that the antiestrogen cannot be inhibiting the cells solely by a mechanism which involves competition with estrogen for receptor sites. We also reject the idea that antiestrogens are simply toxic to the cells. We have demonstrated in Fig. 1 and Table 1 that antiestrogen effects are blocked by simultaneous addition of estradiol. We have previously shown that antiestrogen effects, once established are reversible by estrogen administration [10]. Our previous results have also suggested that cells lacking estrogen receptor are unresponsive to antiestrogens [10] and *in vivo* their effects are primarily on estrogen target tissues [17]. On the other hand, Clark[18] and Katzenellenbogen[19] and their co-workers have shown that antiestrogens such as Nafoxidine (Upjohn U11, 100A) can bind to estrogen receptors and then translocate to the nucleus of the cell where binding appears to differ significantly from

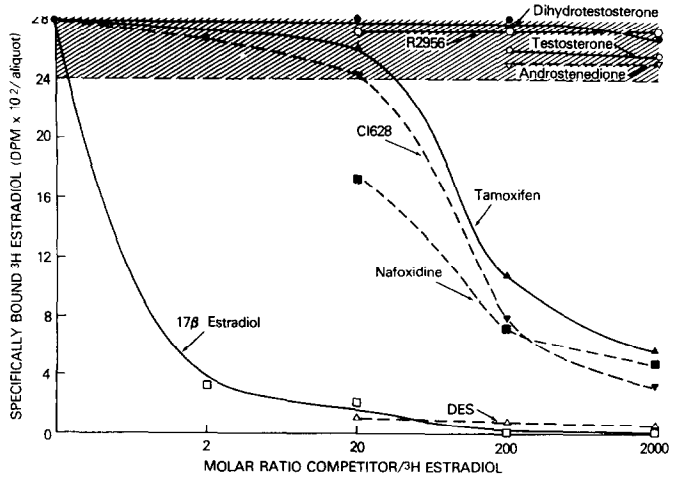

Fig. 3. Binding specificity of the estrogen receptor in a cytoplasmic extract prepared from a breast cancer in a 62 year old post-menopausal female. Methods are identical to those outlined in Fig. 2.

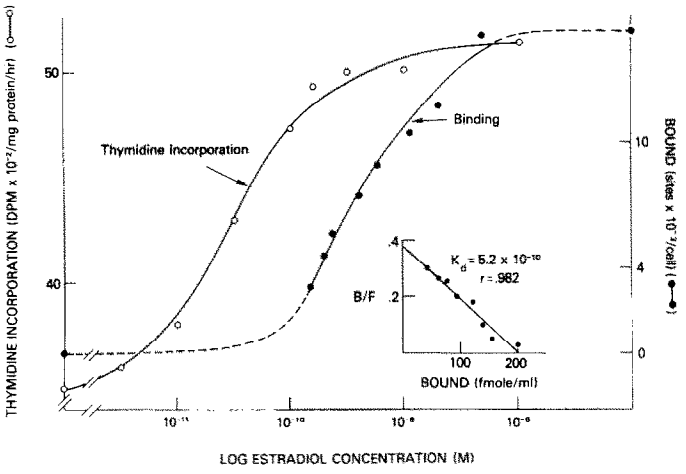

Fig. 4. Comparison of binding of [^{3}H]-17β-estradiol to receptor sites in MCF-7 cells and stimulation of [^{14}C]-thymidine incorporation by estrogen. Binding is measured at 37°C to intact cells as described in Methods. Thymidine incorporation is measured as previously described in the legend of Fig. 1. The dotted line in the binding curve represents the calculated extension of the Scatchard plot shown in the inset to higher and lower B/F values.

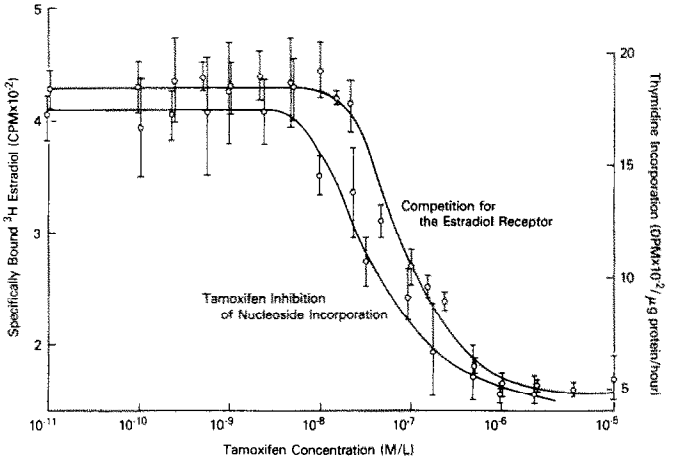

Fig. 5. Comparison of competition of Tamoxifen (ICI 46474) with [^{3}H] 17β-estradiol for estrogen receptor sites and inhibition of [^{3}H]-thymidine incorporation by Tamoxifen. Cytoplasmic extracts from MCF-7 human breast cancer are incubated overnight at 0°C with 10^{-9} M [^{3}H]-17β-estradiol and concentrations of unlabelled Tamoxifen as shown. Following incubation, specifically bound [^{3}H]-estradiol is determined by dextran coated charcoal separation of protein bound and free steroid. Inhibition of [^{3}H]-thymidine incorporation by Tamoxifen is as described in Fig. 1. Results are means of quadruplicate determinations $\pm$ 1 standard deviation for both binding and incorporation data.

Table 1. Effects of 17β-estradiol and tamoxifen (ICI 46474) on [^{3}H]-thymidine incorporation into acid insoluble material in ZR75-1 human breast cancer

Addition	Thymidine incorporation (d.p.m./μg protein/h $\pm$ 1 S.D.)
0	7.1 $\pm$ 0.8
17β-Estradiol 10^{-8} M	12.2 $\pm$ 1.3
Tamoxifen 10^{-6} M	3.8 $\pm$ 0.6
17β-Estradiol 10^{-8} M + Tamoxifen 10^{-6} M	11.6 $\pm$ 1.4

Incubation conditions and precursor incorporation were as described in the legend to Fig. 1.

that of the normal [estrogen–estrogen receptor] complexes. We therefore hypothesize that in these estrogen responsive breast cancer cell lines in tissue culture, [antiestrogen–estrogen receptor] complexes may bind to chromatin and decrease transcription of key DNA segments below control levels, and that perhaps interaction of [estrogen–estrogen receptor] complexes with these same sites increases transcription above control levels. Thus, estrogen receptor would be necessary for expression of antiestrogen effects, but antiestrogen effects would be seen even in the absence of estrogen.

We have previously shown that 10–14 h are required following estrogen administration before any

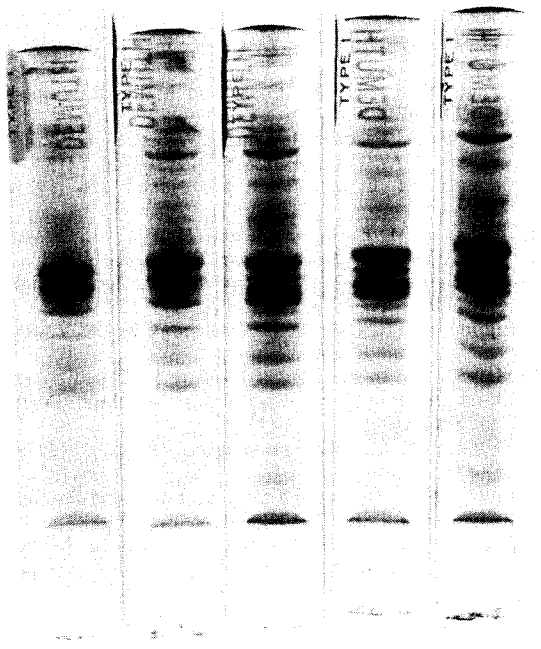

Fig. 6. SDS 7.5% polyacrylamide gels of cytoplasmic proteins in MCF-7 human breast cancer cells following various periods of hormonal stimulation. Techniques are given in Methods. Gels shown represent from left to right cytoplasmic extracts from cells treated with 10^{-8} M 17β-estradiol for 0, 2, 5, 14 and 24 h respectively.

effects on overall nucleoside or amino acid incorporation can be seen [9, 10]. However, it appeared reasonable to hypothesize that synthesis of some proteins, perhaps subserving some regulatory role, might occur earlier. We therefore studied the effects of estrogen on cytoplasmic proteins examined on SDS 7.5% polyacrylamide gels at various times following the addition of 10^{-8} M 17β-estradiol. Results are shown in Fig. 6. At 5 h following estradiol stimulation under serum free conditions, at least one new band is readily apparent which remains on gels of cytoplasmic extracts prepared after 14 and 24 h of hormone stimulation. Other changes in the pattern of proteins synthesized are also apparent. Thus, while gross changes in macromolecular synthesis may not be overt for many hours, this experiment suggests that events are occurring earlier, at least some of which may conceivably be of regulatory importance.

In many of the experiments previously described, the effects of hormone on thymidine incorporation have been studied. We therefore felt that it would be appropriate to examine steps involved in the pathway leading from thymidine in the medium to polymerized nucleoside in DNA. Thymidine kinase catalyzes the first step in the process, namely, phosphorylation of thymidine to thymidylic acid. Activity of this enzyme has been shown to be closely linked to hormonal stimulation of cell growth in other tissues including estrogen dependent rat mammary carcinomas [20].

The effects of various concentrations of estrogen on thymidine kinase activity and thymidine incorporation in human breast cancer in tissue culture are shown in Fig. 7. Two points should be noted. First, the extreme sensitivity of both parameters to estradiol. Second 3×10^{-11} M 17β-estradiol reproducibly stimulates thymidine incorporation in these cells, a fact we have previously noted [10, 11]. Furthermore, stimulation by estradiol of thymidine kinase activity and thymidine incorporation occurs at essentially equivalent concentrations. Thus the two induction curves appear nearly colinear. In additional studies to be reported elsewhere [21] we have shown that thymidine kinase activity is closely correlated with

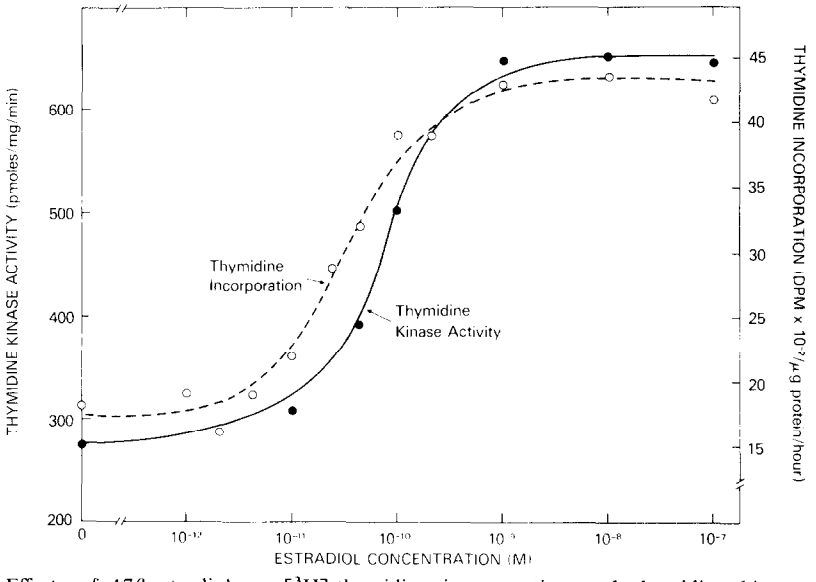

Fig. 7. Effects of 17β-estradiol on [^{3}H]-thymidine incorporation and thymidine kinase activity. [^{3}H]-Thymidine incorporation is assessed as described in Fig. 4. Thymidine kinase activity was measured as outlined in Methods.

levels of thymidine incorporation either inhibited by Tamoxifen or stimulated by estrogen. Finally, induction of thymidine kinase activity either briefly precedes or occurs simultaneously with any increase in thymidine incorporation. Considerably more work will be required on other enzymes involved in DNA synthesis before accurate conclusions may be drawn concerning a possible regulatory role for thymidine kinase activity in mediating estrogen stimulation of cell division in these cells.

In conclusion, we have further characterized two cell lines derived from human breast cancer. Both these cell lines have estrogen receptor and are responsive to physiologic concentrations of estradiol. Antiestrogens inhibit precursor incorporation and growth. Specific protein products stimulated by estrogen administration are identifiable. We therefore feel that such systems should prove quite fruitful for the study of estrogen action *in vitro* generally, and in particular the mechanisms by which estrogen stimulates the growth of human breast cancer.

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DISCUSSION

Jungblut. Would you agree to the following interpretation of your data: estradiol helps to activate what we call a receptor, but this process is not strictly hormone-dependent. Receptor, activated without the aid of estradiol, maintains the growth of the control culture which ceases after the regulating protein is poisoned? My second question is simpler: did you check if a reduction of estrone to estradiol occurs in your cell line?

Lippman. I certainly agree and have said in print that the first hypothesis that you put forward is entirely reasonable. We have examined steroid interconversion of E_1 to E_2 and E_2 to E_1, both occur over the 24 h incubation to approximately 20% of the original steroid. Furthermore the cell lines are capable of making more polar steroids, possibly sulfates or glucuronides and we are currently studying such transformations.

Liddle. I wonder if you can tell us whether any of these estrogen antagonists have activity in whole organisms, either people or experimental animals with breast tumours?

Lippman. There are many people in this room who could comment on it better than I. But all these anti-estrogen compounds have been shown to be active in many animal system in nafoxidine... tamoxifine? are active in human breast cancer.

King. You showed a two-fold increase in thymidine incorporation by 48 h but no change in cell number until 4 days later. Could the thymidine effect be unrelated to the proliferative response?

Lippman. I think that's entirely reasonable.

Clark. This is a question that pertains to the whole philosophy of doing cell culture. I am in favor of doing it and I wish I had a good system like you seem to have. But I always have one problem in my mind, that is: you obtain these cells by doing a lot of screening and a lot of looking and they were not easy to find. Is that correct?

Lippman. That's correct.

Clark. It has been our experience working with Dr. Kohler's group. It is possible that totally incorrect interpretations might be made because you have finally found peculiar cells that do not do what all the rest of the cells in the world do.

Lippman. Well, I think the significance of drawing conclusions from any model system, particularly a tissue culture one in which self selection at any level can occur, in which there may be loss of a variety of controls and regulations simply by putting cells in culture or by the cloning techniques we use have been reviewed and extensively considered by a very large number of people and I share your view only to the extent that means one must be very cautious in drawing conclusions. If we have a cell line that doesn't respond to prolactin then we must not conclude that all human breast cancer does not respond to prolactin. However, similarly if we see a response to estrogen then we must use the hypothesis and the experiments that we can generate in such a system to develop testable kinds of concepts in more complex *in vivo* situations. There is no objection in what you are saying. I

think the major thing is that one can do in this sort of mixed experiment that are really extraordinarily difficult to do *in vivo* and that this may allow development of entirely different levels of sophistication. One must bear in mind that it might not apply.

Crabbé. In your concluding slide you state that high concentrations of estradiol can kill the cells through a process not involving the receptor system. To what extent is this observed only with estrogens? And a 2nd point how specific is this killing effect with regard to the type of cells being cultured?

Lippman. Since it is known that pharmacological concentrations of estrogen are efficacious in DMBA induced in breast cancer and in human breast cancer we were interested in doing a total dose response curve to estrogen. When we originally did that at concentrations slightly in excess of 10^{-6} molar 17β-estradiol inhibited and eventually killed the cells. We did the control studies to go with that and found that other estrogens for example, 3 methyl ether estradiol, 17α-estradiol were equally good at killing phenomenon without stimulating the cells. We found that nonestrogens etiocholanolone, 5β-DHT could also reproduce this killing phenomenon, we were then able to show that in cells that lacked estrogen receptor we could also induce this killing at high concentrations without concomitant stimulation at low concentrations of estradiol and finally we were able to show that other nonmammary lines, hepatoma and rat kidney, were also killed by these high concentrations. So our conclusion was that in our system we were unable to set up a set of conditions whereby we could reproduce what appears to be the specificity of killing by high concentrations of estrogens *in vivo*. To our knowledge nobody has ever used 'a biologically inactive estrogen' like 17α to treat human cancer (but we just have not figured out the conditions to do those experiments)?