[CANCER RESEARCH 38, 4135–4137, November 1978]
0008-5472/78/0038-0000$02.00

# Multiple Sensitivities of Mammary Tumor Cells in Culture[1]

**Jean Yates and Roger J. B. King**

*Department of Hormone Biochemistry, Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, England*

## Abstract

**Cloned cell lines derived from the androgen-responsive Shionogi 115 mouse mammary carcinoma, when cultured in the presence of $3.5 \times 10^{-8}$ M testosterone, retain their responsiveness to testosterone and 5α-dihydrotestosterone and exhibit fibroblast-like morphology; their growth is poorly regulated by cell density. Dexamethasone at $10^{-8}$ M inhibits proliferation of these cells by 30% but stimulates them by 235% at $10^{-6}$ M in the temporary absence of testosterone. Cell growth is little affected by serum concentration. When cultured for 3 to 4 weeks in testosterone-free medium, however, the cells lose their androgen responsiveness and retain the inhibitory but not the stimulatory response to dexamethasone. They also show an increased sensitivity to serum and increased density regulation and change to an epithelial morphology.**

**It is suggested that the loss of sensitivity to androgens, which does not result from absence of androgen receptor, is related in a complex way to the increased sensitivities to serum and density regulation.**

## Introduction

Steroid hormones regulate the proliferation of many normal and neoplastic cells. The early events involving binding of the steroid to specific receptor proteins and the translocation of the complex to the nucleus are well documented (8, 9); events occurring after nuclear entry are, however, poorly understood. During studies on the androgen regulation of S115 mouse mammary tumor cells in culture, an observation was made that provided a potential method for investigating postreceptor events. S115 cells maintained in the presence of androgen retain their androgen responsiveness for many weeks, whereas cells grown in androgen-deprived medium rapidly lose their responsiveness (6). This loss of response is not accompanied by major changes in whole-cell or nuclear receptor levels from which it was concluded that a postreceptor defect existed in these cells (5, 7). Comparison of the androgen-maintained (+A) responsive cells with their unresponsive deprived (−A) counterparts could give some clues as to the locus of the defect and help to define part of the postreceptor pathway. As a first step we decided to study the sensitivities of the +A and −A cells to a number of agents involved in the regulation of cell proliferation. It was reasoned that, if the putative defect occurred at a point before that at which the other agents fed into the pleiotypic, proliferative mechanism, no difference in sensitivity to those agents would be seen. If, however, the defect occurred at a site common to other agents, sensitivity to those agents would be reduced. This paper describes experiments to measure the sensitivity of +A and −A cells to glucocorticoids, serum, cell density, and prostaglandins plus insulin.

## Materials and Methods

**Cell Culture.** Stock cells were routinely cultured in a humidified atmosphere of 5% carbon dioxide in air at 37° in Dulbecco's modified Eagle's medium supplemented with 2% fetal calf serum (Gibco Bio-Cult, Glasgow, Scotland) and 40 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Sigma Chemical Co., London, England). The growth medium for androgen-maintained (+A) cells also contained $3.5 \times 10^{-8}$ M testosterone (0.01 μg/ml). Androgen-deprived (−A) cells were derived from the same +A cloned line by plating the cells in medium without testosterone (7). −A cells were maintained in this way for at least 6 weeks prior to their use in growth experiments. Cells were subcultured at weekly intervals in 90-mm plastic culture dishes (Sterilin, Ltd., Teddington, Middlesex, England).

**Hormones.** Testosterone and 5α-dihydrotestosterone (5α-androstan-17β-ol-3-one) from Steraloids, Ltd., Croydon, England, and dexamethasone (Sigma) were dissolved in absolute ethanol at 1000 times the concentration finally required. An aliquot of this stock solution was added to the culture medium before being added to the cells. The same concentration of ethanol was added to control plates.

**Cell Growth Experiments.** Cells were suspended in 0.05% trypsin buffered with 0.02% EDTA (pH 7.2), counted on a hemocytometer, and plated at $1 \times 10^5$ cells/50-mm plastic culture dish in 5 ml medium containing 2% serum unless otherwise stated. After 24 hr the medium was changed to steroid-containing medium which was not changed again during the experimental period. For experiments in which the serum concentration was varied, medium containing the serum concentration required was prepared before plating the cells. For long-term growth curves, the medium was changed after 24 hr and on every following third day. Cell numbers were estimated from triplicate Coulter counter readings of trypsinized cell suspensions diluted with Isoton (Coulter Electronics Ltd., Harpenden, England). All cell counts were done on triplicate plates, and results were calculated as the mean ± S.E.

## Results

The response of S115 cells to culture for 4 days in the presence of varying concentrations of testosterone is shown in Chart 1. Similar dose-response curves (not shown) were also obtained for 5α-dihydrotestosterone. Androgen-maintained cells showed a 7-fold increase in cell numbers at concentrations of testosterone greater than $10^{-9}$ M while

[1] Presented at the John E. Fogarty International Center Conference on Hormones and Cancer, March 29 to 31, Bethesda, Md.

androgen-deprived cells were completely unresponsive to this range of testosterone levels. The concentration of 3.5 × $10^{-8}$ M testosterone used in routine subculture of the +A cells and in all experiments in which parameters other than androgen concentration were varied is within this plateau region.

During the course of these experiments, it was noted that the glucocorticoid dexamethasone had a biphasic effect on +A cells temporarily grown in the absence of testosterone (Chart 2*b*); inhibition of growth occurred at $10^{-8}$ M dexamethasone whereas higher concentrations stimulated growth. In 3 experiments the inhibition of +A cells brought about by $10^{-8}$ M dexamethasone was 31 ± 5%. The mean ± S.E. stimulation by $10^{-6}$ M dexamethasone was 235 ± 20%.

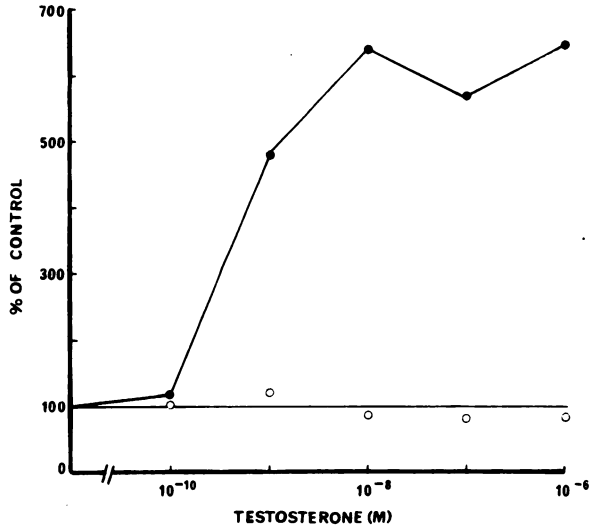


Chart 1. The effect of testosterone on the growth of androgen-maintained (●) and androgen-deprived (○) cells. As described in "Materials and Methods," 1 × $10^5$ cells were cultured for 4 days in the stated concentration of testosterone. Results are expressed as a percentage of the cell numbers in control plates which were 4.1 × $10^{-5}$ for +A cells and 2.6 × $10^{-5}$ for −A cells.

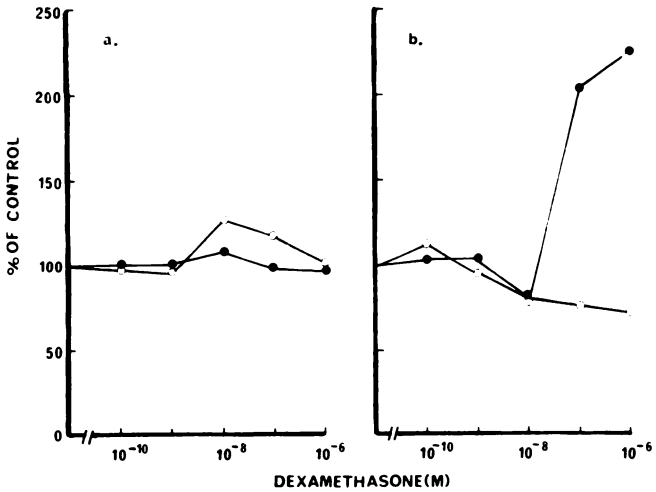


Chart 2. The effect of dexamethasone on the growth of androgen-maintained (●) and androgen-deprived (○) cells in the presence (*a*) or absence (*b*) of 3.5 × $10^{-8}$ M testosterone. As described in "Materials and Methods," 1 × $10^5$ cells were cultured for 6 days in the stated concentration of dexamethasone. Results are expressed as a percentage of cell numbers in control plates.

There was a progressive inhibition of proliferation of −A cells reaching 25 ± 3% at $10^{-6}$ dexamethasone. When testosterone (3.5 × $10^{-8}$ M) was added, the proliferation of +A and −A cells was not markedly affected by any concentration of dexamethasone (Chart 2*a*). Cortisol gave similar but less reproducible results. This is probably due to the presence of corticosteroid-binding globulin in the fetal calf serum.

Androgen-maintained and -deprived cells also show a marked difference in their sensitivity to serum concentration (Chart 3). The growth of −A cells in both the presence and the absence of 3.5 × $10^{-8}$ M testosterone is considerably stimulated by increased concentrations of serum, whereas the proliferation of +A cells is influenced to a lesser extent. These results are expressed as cell numbers per plate rather than as percentage of control value, because it was believed that the lowest serum concentration used (2%) did not represent a true control value in the same way as that used in experiments in which steroid concentrations were varied. The corresponding control would have involved the use of serum-free medium which would have severely reduced the plating efficiency of the cells. Plating efficiency was not affected by serum concentration.

Longer-term culture of +A cells in the presence of 3.5 × $10^{-8}$ M testosterone and of −A cells in the absence of testosterone during which the medium was changed at 3-day intervals to ensure adequate supply of nutrients and testosterone is illustrated in Chart 4. The proliferation rate of the +A cells over the rapid growth part of the curve is approximately 3 times that of −A cells, and the final cell density reached before the cells detach from the plate is much higher for +A cells. The +A cells have a fibroblastic appearance and grow in an irregular manner, often overlapping adjacent cells. Androgen-deprived cells, on the other hand, show an epithelial morphology and grow to a confluent monolayer.

Prostaglandin $F_{2\alpha}$ at concentrations of 100 to 500 ng/ml in the presence of insulin (50 ng/ml) when added to the cells in the absence of testosterone showed a variable effect on the proliferation of both cell types, and no definite stimulation or inhibition could be detected. Incidental ob-

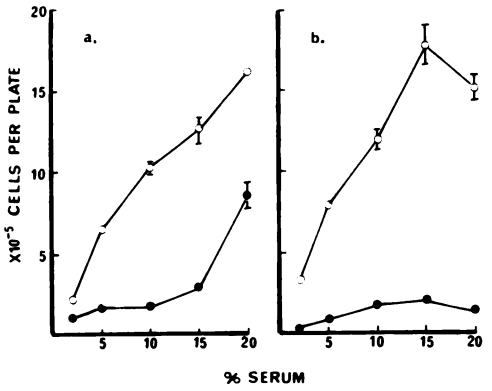


Chart 3. The effect of serum concentration on the growth of androgen-maintained (●) and androgen-deprived (○) cells in the presence (*a*) or absence (*b*) of 3.5 × $10^{-8}$ M testosterone. We cultured 1 × $10^5$ cells for 4 days. Each *point* represents the mean count of triplicate plates; *bars*, S.E. For clarity *error bars* are not shown when they are so small as to obscure the symbols.

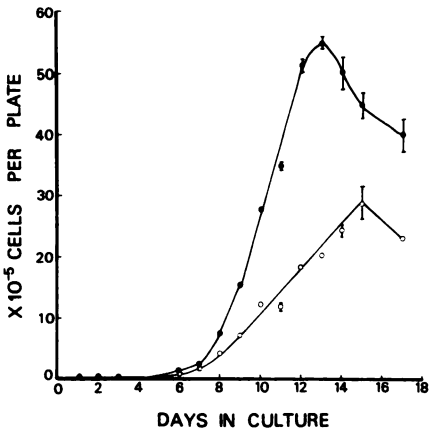


Chart 4. Growth curves of androgen-maintained cells in the presence of $3.5 \times 10^{-8}$ M testosterone (●) and of androgen-deprived cells in the absence of testosterone (○). We plated 50,000 cells in 50-mm culture dishes. Each *point* represents the mean count of triplicate plates; *bars*, S.E. For clarity *error bars* are not shown when they are so small as to obscure the symbols.

servations during the course of these experiments indicated that +A cells are more sensitive than −A cells to such influences as extremes of pH, elevated temperature, and the presence of ethanol in the culture medium.

## Discussion

The loss of androgen sensitivity resulting from 3 or 4 weeks culture of S115 mouse mammary tumor cells in androgen-free medium cannot be due to genotypic selection and is not accompanied by detectable changes in nuclear or cytoplasmic androgen receptors (5, 6). The mechanisms involved in the loss of responsiveness are not known. The results presented in this paper indicate that, when S115 cells lose their androgen responsiveness, their sensitivities to other agents change in a complex manner.

Dexamethasone inhibits growth of androgen-maintained cells at low concentrations, whereas higher concentrations (greater than $10^{-8}$ M) are stimulatory. Androgen-deprived cells retain the inhibitory but not the stimulatory response to dexamethasone. Addition of a maximally stimulating concentration of testosterone to the androgen-maintained cells abolishes the dexamethasone effect. This observation indicates that dexamethasone and testosterone act via a common pathway, but it remains to be shown whether this occurs at the level of receptor binding or at a postreceptor stage.

Loss of androgen sensitivity is accompanied by an increased sensitivity to both serum and cell density. The serum effect is observed in both the presence and the absence of testosterone. It is not possible to explain this effect by the presence of androgens or androgen-binding proteins in the serum; therefore we conclude that the androgen-deprived cells are more sensitive to as yet undefined factors in the serum. Given the close relationship between serum sensitivity and cell density (2, 4), the changes in sensitivity noted here may be related. However, the relationship cannot be a simple one because the published data obtained with fibroblasts indicate that increased serum sensitivity is associated with increased limiting cell density (1, 10); our S115 cells exhibit the opposite effect.

Androgen-maintained cells temporarily grown in the absence of testosterone have an epithelial appearance which changes to a fibroblastic morphology on stimulation with testosterone (3, 11). In the present experiments, it was noted that morphologies of the androgen-maintained and deprived cells were different. Certainly, an examination of the cellular properties that influence growth and morphology will be rewarding.

It is clear from the results presented here that the model proposed in the "Introduction" in which the site of action of various agents affecting proliferation might be determined by determining their dose-reponse curves in androgen-maintained and deprived cells is too simple. The model does not predict the observed increase in sensitivity to stimuli such as serum and cell density. The elucidation of the temporal relationship between the loss of androgen sensitivity and the increase in sensitivity to both serum and cell density together with the appearance of morphological changes will help to resolve this problem.

## References

1. Castor, L. N. Control of Division by Cell Contact and Serum Concentration in Cultures of 3T3 Cells. Exptl. Cell Res., *68:* 17–24, 1971.
2. Clarke, G. D., Stoker, M. G. P., Ludlow, A., and Thornton, M. Requirement of Serum for DNA Synthesis in BHK 21 Cells: Effects of Density, Suspension and Virus Transformation. Nature, *227:* 798–801, 1970.
3. Desmond, W. J., Wolbers, S. J., and Sato, G. Cloned Mouse Mammary Cell Lines Requiring Androgens for Growth in Culture. Cell, *8:* 79–86, 1976.
4. Dulbecco, R. Topoinhibition and Serum Requirement of Transformed and Untransformed Cells. Nature, *227:* 802–806, 1970.
5. King, R. J. B. Studies on the Regulation of Cell Proliferation in Culture by Steroids. *In*: J. Dumont and J. Nunez (eds.), Hormones and Cell Regulation, European Symposium, Vol. 2, pp. 15–36. Amsterdam: North-Holland Publishing Co., 1978.
6. King, R. J. B., Cambray, G. J., Jagus-Smith, R., Robinson, J. H., and Smith, J. A. Steroid Hormones and the Control of Tumor Growth: Studies on Androgen-responsive Tumor Cells in Culture. *In:* J. R. Pasqualini (ed.), Receptors and Mechanism of Action of Steroid Hormones, Part 1, pp. 41–84. New York: Marcel Dekker, Inc., 1976.
7. King, R. J. B., Cambray, G. J., and Robinson, J. H. The Role of Receptors in the Steroidal Regulation of Tumor Cell Proliferation. J. Steroid Biochem., *7:* 869–873, 1976.
8. King, R. J. B., and Mainwaring, W. I. P. Steroid-Cell Interactions. London: Butterworths, 1974.
9. Munck, A. General Aspects of Steroid Hormone-Receptor Interactions. *In:* J. R. Pasqualini (ed.), Receptors and Mechanism of Action of Steroid Hormones, Part 1, pp. 1–40. New York: Marcel Dekker, Inc., 1976.
10. Oey, J., Vogel, A., and Pollack, R. Intracellular Cyclic AMP Concentration Responds Specifically to Growth Regulation by Serum. Proc. Natl. Acad. Sci. U. S., *71:* 694–698, 1974.
11. Robinson, J. H., and Smith, J. A. Density-dependent Regulation of Proliferation Rate in Cultured, Androgen-responsive Tumor Cells. J. Cellular Physiol., *89:* 111–122, 1976.

# Multiple Sensitivities of Mammary Tumor Cells in Culture

Jean Yates and Roger J. B. King

*Cancer Res* 1978;38:4135-4137.