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EFFECT OF RETINOIC ACID AND 4-HYDROXYTAMOXIFEN ON HUMAN BREAST CANCER CELL LINES

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Abstract—Using established breast cancer cell lines in a cell culture model we studied the growth effect of retinoic acid (RA) alone or in combination with the antiestrogen 4-hydroxytamoxifen (OHT). Cytoplasmic ^{3}HRA binding sites were determined by sucrose density gradient centrifugation analysis. Of the three cell lines Hs578T, BT 20, and 734 B only the last showed a significant amount of specific RA binding (10^5 sites/cell). This cell line showed a dose dependent decrease in proliferation after a long-term incubation with RA whereas the ^{3}H-thymidine uptake was highly significantly increased after incubation with 10^{-6} M of RA for 20 hr. Growth inhibition was not further increased by the addition of OHT (10^{-6} M), but the increase in thymidine incorporation due to RA was neutralized by OHT. Hs578T and BT 20 cells were not affected by any of the treatments. The different action of RA on proliferation and thymidine incorporation suggests a cell cycle specific mechanism.

Vitamin A (retinol, Vit. A) is an essential nutrient which plays an important role in many physiological functions such as in maintenance of rod vision and in the differentiation of epithelial tissues. Whereas the participation of Vit. A in the process of visual excitation is well known, the molecular action in cellular differentiation has not been clarified. In addition to night blindness, Vit. A deficiency results in disorders of differentiation such as metaplasia or hyperplasia [1]. Cancer, which may be considered as a lack of differentiation, has been shown to be influenced by retinoids. Individuals with higher than average blood retinol levels were found to have a decreased risk of developing cancer [2]. On the other hand, laryngeal carcinoma patients often have low blood retinol values as well as disturbed dark adaption, which is a sensitive indicator of Vit. A deficiency [3].

Retinoids reduce the efficacy of many carcinogens to induce epithelial tumors [4, 5]; a growth inhibitory action *in vitro* on several established cell lines has also been reported [6–9].

The aim of the present study was to investigate the effects of retinoic acid (RA),† one of the most potent retinoids [10], on three established mammary cancer cell lines. The following questions were posed: Is the cell proliferation inhibitory action of RA associated with the presence of a cytoplasmic RA binding protein (CRABP)? Does RA change the responsiveness of the cells to antiestrogens like tamoxifen? This second point is of interest because Moon *et al.* have recently shown that ovariectomy and retinoids exhibit a synergistic, and tamoxifen and retinoids an additive, effect on the inhibition of carcinogenesis *in vivo* [4, 11]. Combinations of RA and 4-hydroxytamoxifen (OHT) were used to study the effects elicited by these compounds on cell proliferation. The natural metabolite of tamoxifen, OHT, was chosen for its high antiestrogenic and tumour growth inhibitory action [12].

MATERIALS AND METHODS

Retinoids. RA and ^{3}H-RA (7.5 Ci/mmole) were a gift from Hoffmann–La Roche. The procedures with the retinoids were performed in subdued light. 10^{-2} M RA solutions in DMSO were used to prepare the cell culture media. The purity of the solutions was checked by thin layer chromatography.

Cell culture. The human mammary carcinoma cell lines BT 20, Hs578T and 734 B were a generous gift from Gertrude C. Buehring (School of Public Health, University of California, Berkely). Hs578T was originally characterized and supplied by A. J. Hackett (Naval Biomedical Research Laboratory, Oakland). BT20 and Hs578T cells are estrogen receptor negative and not estrogen dependent [13, 14]. 734B was found by us to be estrogen receptor positive and estrogen dependent (unpublished data). Cells were grown in minimum essential medium with Earle's salts (MEM), supplemented with 10% fetal calf serum (FCS), L-glutamine (2 mM), gentamycin (50 μg/ml) and amphotericin B (5 μg/ml). For the stock culture, cells were grown as monolayer in T75 plastic flasks (Falcon Plastics Company, Oxnard, California), under humid atmosphere and 5% CO_2 in air at 37°. Cells were harvested with trypsin (0.05%)–EDTA (0.02%) in saline and stored in liquid nitrogen in MEM containing 10% FCS and 10% DMSO.

Analysis of the cytoplasmic retinoic acid binding protein (CRABP). The cells which had been stored

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† Abbreviations: RA, retinoic acid; OHT, 4-hydroxytamoxifen; CRABP, cytoplasmic RA binding protein; DMSO, dimethyl sulfoxide; MEM, minimum essential medium; FCS, fetal calf serum; TCA, trichloracetic acid; S, Svedberg unit.

in liquid nitrogen were homogenized in phosphate buffer (0.05 M, pH 7.4, 0.1% Triton X100) using a glass homogenizer. All operations were performed at 4° to eliminate the possibility of degradation of the binding protein. Cytosol was prepared and sucrose gradient centrifugation was performed as previously reported [15].

Thymidine incorporation. Cells were seeded as 1 ml suspensions and allowed to attach for 24 hr. Thereafter the medium was changed and the fresh medium was supplemented with RA (10^{-6}, 10^{-8}, 10^{-10} M) or OHT (10^{-6} M) or RA plus OHT (each 10^{-6} M). In the control group the cells were grown in medium without supplements. After 4 hr, and after 20 hr, the cells were incubated for one hour in the presence of 0.5 μCi/ml ^{3}H-thymidine. At the end of each incubation, the cells were washed twice in ice-cold 0.15 M sodium chloride, twice in 5%-trichloracetic acid (TCA) and then once in ethanol. Cells were lysed by incubating them with 0.5 ml of 0.15 M potassium hydroxide for one hour at 50°. Aliquots were placed in 8 ml of scintillation fluid and the radioactivity was determined in a liquid scintillation counter. Each value was obtained by 12 repetitions.

Growth effects. Cells from stock flasks were suspended with trypsin (0.05%)-EDTA (0.2%) and seeded in Nunc 24 well tissue culture plates (Nunc 69590, Roskilde, Dk) as 1 ml suspensions in MEM 10% FCS. The cells were allowed to attach for 24 hr, and the medium was changed to the appropriate concentration of retinoids and/or OHT.

The medium was changed every 2 to 3 days.

To construct growth curves, the cell number was determined using an electronic particle counter (Coulter Electronics Ltd., Dunstable, U.K.) at the beginning of each experiment (day 0, 12 wells) and at suitable successive time intervals (6 wells per group per day).

To demonstrate the dose dependence of the retinoid effect, 3 RA concentrations (12 wells per concentration) were included in the above experiment and harvested and enumerated at the last timepoint of the growth curves.

Statistical evaluation. The treatments were compared using the Kruskal–Wallis one way analysis of variance by ranks and the Wilcoxon test. The growth curves were evaluated by the nonparametric variance analysis from Wilcoxon and Wilcox [16].

RESULTS

The sucrose gradients (Fig. 1) demonstrate that only the cell line 734 B possessed a significant amount of CRABP. The 2S peak corresponds to 2.0 pmole/mg protein or 106,000 binding sites per cell. Hs578T and BT 20 showed no specific binding sites for RA in the cytosol. The 4S binding is assumed to be nonspecific as it cannot be inhibited by an excess of unlabeled RA. The influence of RA after a short incubation period was estimated by the rate of ^{3}H-thymidine incorporation into the TCA insoluble fraction. The two CRABP negative cell lines BT 20 and Hs578T were not affected significantly by RA (Figs. 2A and B). Both cell lines also showed no significant change in the ^{3}H-thymidine uptake when incubated with OHT (Figs. 3A and B). The combination of the two drugs, OHT and RA, was not able to generate any significant effect (Figs. 3A and B). The CRABP positive cell line 734 B showed an increased ^{3}H-thymidine incorporation after a 20 hr incubation with RA ($P < 0.001$, Fig. 2C). This activation showed a strong dose dependence. OHT, however, decreased the incorporation of radioactivity into the TCA insoluble fraction after a 4 hr treatment and even more pronounced after 20 hr (Fig. 3C). Simultaneous incubation of OHT and RA led to a neutralisation of the two effects.

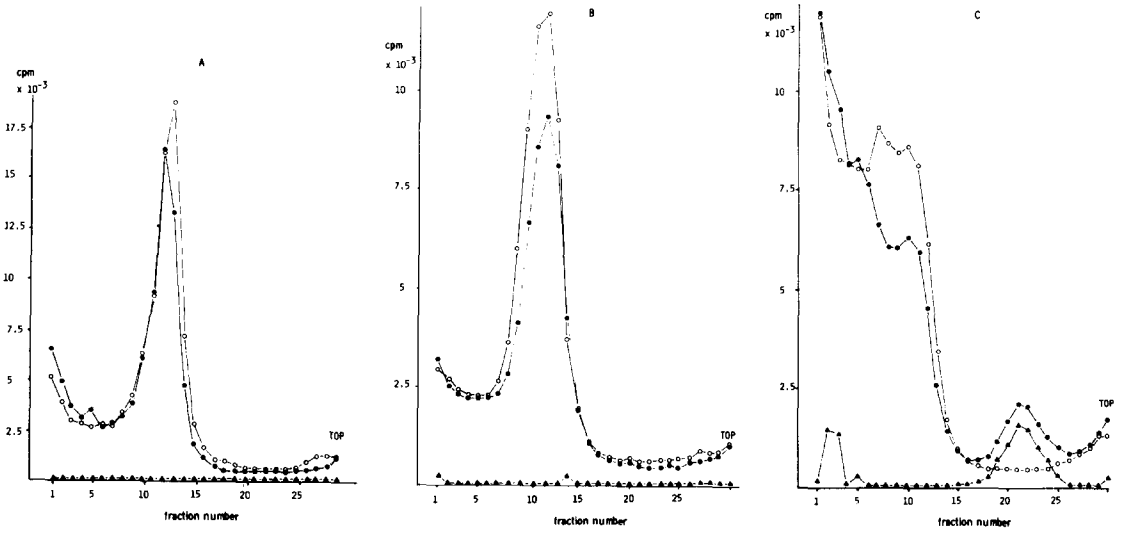

Fig. 1. Sucrose density gradient analysis of the cytoplasmic ^{3}H RA binding protein in Hs578T (A), BT20 (B) and 734B (C) cells. Cytosols were incubated with 400 nM ^{3}H RA alone (● total binding) or with a 100 fold excess of unlabeled RA (○ nonspecific binding). ▲ Specific RA binding (total minus nonspecific) The marker proteins human serum albumin (4.6S) and myoglobin (2S) were found in fraction 10–13 and 19–23 respectively.

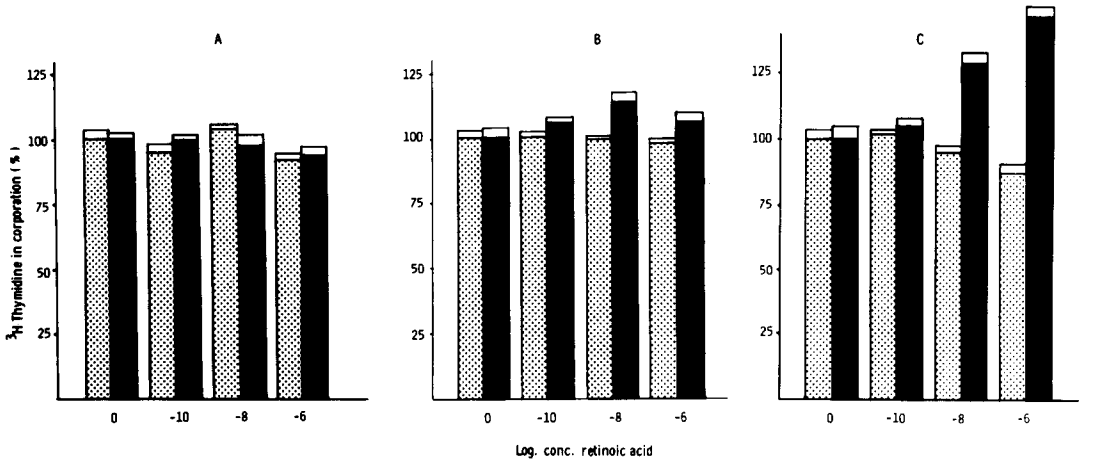

Fig. 2. Dose–response curves showing the effects of RA on the incorporation of ^{3}H-thymidine in Hs578T (A), BT20 (B) and 734B (C) cells. Results are presented as the mean of 12 determinations + S.E.M. The radioactivity measured in the control group without RA was set as 100%. The dotted bars show the effects after a 4 hr incubation, the black bars after a 20 hr incubation with RA.

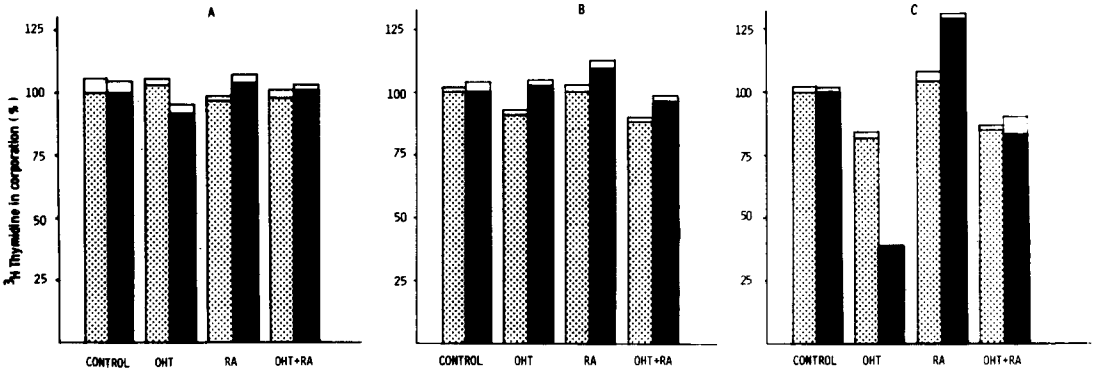

Fig. 3. Effects of RA (10^{-6} M), OHT (10^{-6} M) and their combination in relation to a control group without additives on the incorporation of ^{3}H-thymidine in Hs578T (A), BT20 (B) and 734B (C) cells. Results are presented as the mean of 12 determinations + S.E.M. The radioactivity measured in the control group was set as 100%. The dotted bars show the effects after a 4 hr incubation, the black bars after a 20 hr incubation.

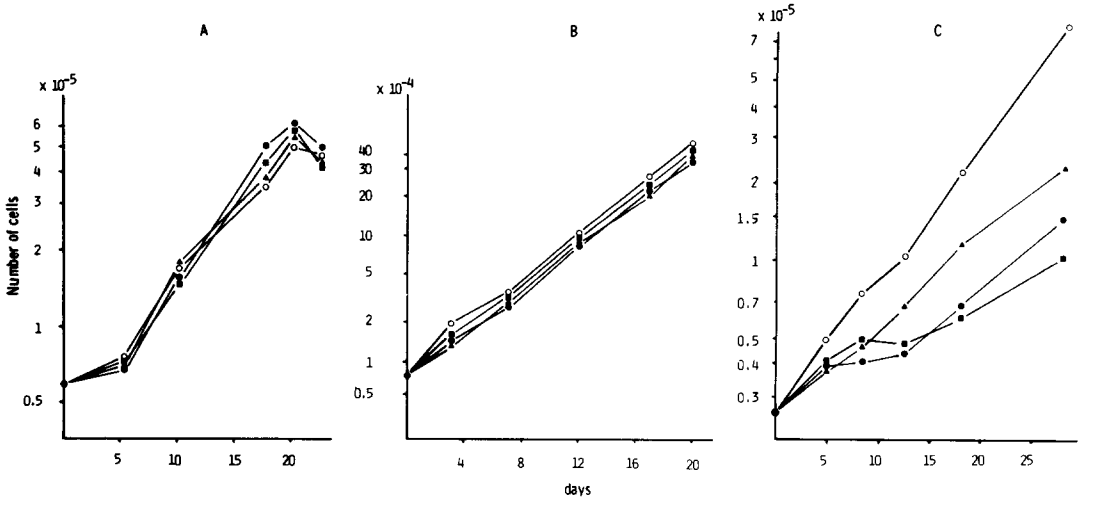

Fig. 4. Growth curves of Hs578T (A), BT20 (B) and 734B (C) cells. The cells were incubated with RA (10^{-6} M, ■), OHT (10^{-6} M, ▲), RA + OHT (each 10^{-6} M, ●) and with neither RA nor OHT as a control (○). The results are plotted as the mean of 6 determinations. The standard deviation was always lower than 15% and is not shown in this figure to maintain the clarity.

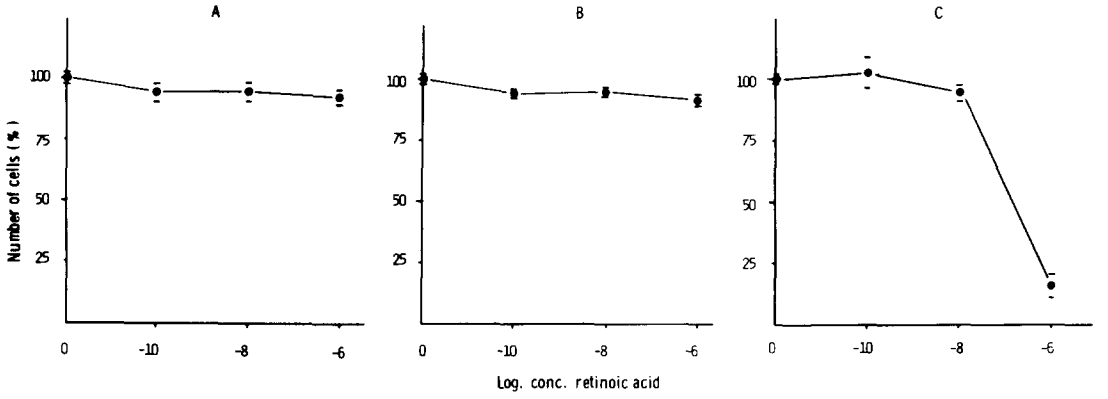

Fig. 5. Dose dependence of the effect of RA on the proliferation of Hs578T (A), BT20 (B) and 734B (C) cells. Results are plotted as the mean of 12 determinations ± S.E.M. The cell number of the control group was set as 100%. The cells were harvested after 20–25 days of treatment.

Growth curves were constructed to demonstrate the effects of RA on cell proliferation during long term incubation periods. BT 20 (Fig. 4A) and Hs578T (Fig. 4B) were not affected by RA either alone or in combination with OHT. In addition, the dose–response curves showed no growth inhibition by RA for these two cell lines (Figs. 5A and B). The proliferation of 734B cells, however, was significantly reduced by RA ($P < 0.001$) and OHT ($P < 0.01$) (Fig. 4C). RA showed a greater inhibitory action than OHT ($P < 0.05$). The effect was not increased by applying the two drugs together. The curves under RA treatment and under RA + OHT treatment were statistically indistinguishable. The decreased proliferation after RA treatment was dose-dependent (Fig. 5C).

DISCUSSION

In our experimental system only the CRABP positive cell line 734B was affected by RA. BT 20 and Hs578T showed no effect when treated with RA. All cell lines are derived from human breast cancer, however during the long period they have been maintained in culture may lead to considerable deviations from the tumour they originate. It is of interest to note that Lacroix *et al.* [8] have found CRABP in Hs578T and also a growth inhibition by RA. In repeated experiments in our laboratory we were not able to reproduce these results; one explanation could be that our cells have been transformed into RA insensitive cells through loss of their RA binding sites. Our results raise the possibility of the existence of at least 2 Hs578T cell lines: one possessing a CRABP which can be inhibited by RA and a second cell line deficient in CRABP which is unresponsive to RA treatment. In agreement with other authors [17, 18], these results suggest the requirement of the CRABP for effects of RA. The RA action is assumed to be mediated by translocation of CRABP into the nucleus and binding to chromatin, a process which would be similar to the mode of action of steroid hormones. The ^{3}H-thymidine uptake in the 734B cell line was increased after a 20 hr incubation period in the presence of RA; however, cell proliferation was not increased. This contradictory effect may be explained by an enhanced DNA synthesis and stimulation of the cells to begin mitosis but with a block at the G2 or M-phase. If this is true, this may explain why retinoids are able to potentiate the effect of X-rays or certain cytostatic drugs on tumour regression [19, 20]. OHT showed complete neutralisation of the increased ^{3}H-thymidine incorporation invoked by RA but was not able to potentiate the growth inhibitory action of RA. Both RA and OHT have their own binding entities but they may influence the cell cycle in an interdependant manner, because RA + OHT resulted in the same growth inhibition as did RA alone. The different action of the combination of RA and OHT in inhibition of carcinogenesis and inhibition of cancer cell proliferation suggests that there are two distinct mechanisms and they should not be considered as being equivalent.

RA or its analogues may represent a new and interesting approach in the treatment of breast cancer even though an initial clinical trial gave discouraging results [21]. However, the measurement of CRABP may prove valuable in predicting tumor responsiveness to RA treatment in a manner similar to the use of estrogen and progesterone receptor measurement in predicting hormone responsiveness in breast tumors.

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