

## EFFECTS OF DIHYDROTESTOSTERONE AND HYDROXYFLUTAMIDE ON ANDROGEN RECEPTORS IN CULTURED HUMAN BREAST CANCER CELLS (EVSA-T)

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**Summary**—The purpose of our study was to evaluate the effects of 5 $\alpha$ -dihydrotestosterone (DHT) and hydroxyflutamide (HF), alone or in combination, on androgen receptor (AR) dynamics and on cellular growth in cultured breast cancer cells (EVSA-T). The incubation of cells with DHT increased the concentration of nuclear AR after 24 and 48 h. HF was also able to promote the nuclear accumulation of AR after 24 and 48 h of treatment. When HF-treated cells are incubated with DHT, the nuclear AR concentration is lower than that found in cells treated with DHT alone. We conclude that HF acts by increasing nuclear accumulation of receptor-antiandrogen complexes. Moreover, DHT stimulates cell growth while HF has an inhibitory effect. Thymidine incorporation in cells also increased after DHT treatment and decreased after HF incubation. The HF-induced inhibition of cell growth persisted both after renewal of the medium and after the addition of DHT to cultures. It may be hypothesized that either DHT is converted to inactive metabolites or that HF exerts a persistent inhibitory effect. In the latter case, the antiandrogen action of HF could be exerted by retention of high levels of antiandrogen in cells or by such a depressed protein synthesis that the renewal of growth is slower than the 48 h period studied.

### INTRODUCTION

The mechanism of cell growth stimulation by androgens is not fully understood. Since androgens are at times used to induce breast cancer regression in women, it is important to determine why the opposite effect is observed in human breast cancer cells in culture [1, 2]. Several cell lines of human breast carcinoma, containing androgen receptors (AR), have been used as models, to study the possible role of androgens in tumor growth [2-5]. Assuming that the proliferative action of androgen is carried out by its direct interaction with intracellular AR, many antiandrogens have been used to compete for the steroid binding to AR. It has been demonstrated that the non-steroidal compound hydroxyflutamide (HF;  $\alpha,\alpha,\alpha$ -trifluoro-2-methyl-4'-nitro-*m*-lactotoluidide) is a potent antiandrogen *in vivo* [6-8] and that it is

also devoid of androgenic effects [9, 10]. However, the exact mechanism of competitive action of HF on receptor binding and on subsequent steps of action in the nuclei is poorly understood. In contrast to the proposed mechanism of action for antiestrogens [11, 12], many studies [13, 14] have suggested that both steroidal and non-steroidal antiandrogens may act by combining with receptors to form complexes which are unable to translocate into nuclei. Recent findings, yielded by studies with antiandrogens, have indicated that these compounds are capable of translocating mouse renal cytosol ARs to nuclei *in vivo* [15, 16]. Moreover, it has been shown that non-steroidal antiandrogens are able to increase the nuclear concentration of antiandrogen-receptor complexes which are biologically inactive [16].

In order to study the interrelationships between androgens and antiandrogens and their effects on AR and cellular growth, we have examined the effect of 5 $\alpha$ -dihydrotestosterone (DHT) and HF on a breast carcinoma cell line, EVSA-T. Being rich in AR and poor in estrogen

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and progesterone receptors, this cell line could be an ideal experimental model to study the mechanism of androgens and antiandrogens on ARs in cancer cells.

### EXPERIMENTAL

Culture medium and fetal calf serum (FCS) were purchased from Flow Laboratories (Ayrshire, England). HF was generously provided by Essex (Italy); DHT was obtained from Sigma Chemicals (St Louis, MO). [ $^3\text{H}$ ]Methyltrienolone ([ $^3\text{H}$ ]R 1881 87 Ci/mmol) and unlabeled methyltrienolone (R 1881) were obtained from New England Nuclear Corporation (Boston, MA).

#### *Cell culture*

The EVSA-T cell line was kindly provided by Dr G. Leclercq from Institut Jules Bordet (Bruxelles, Belgium). These cells were derived from malignant ascitic effusion from a female patient with metastatic breast carcinoma [17]. Cells were maintained in DMEM (Dulbecco's Modified Eagle's Medium), supplemented with 10% FCS 2%, L-glutamine (200 mM), 1% non-essential amino acids and 200  $\mu\text{g}/\text{ml}$  gentamicin. Cells were grown at 37°C in a humidified atmosphere containing 5%  $\text{CO}_2$  in air. The culture medium was renewed twice a week. Subconfluent monolayers were trypsinized weekly, counted, checked for viability by the trypan blue exclusion test and  $6.0 \times 10^6$  cells were plated in 75  $\text{cm}^2$  flasks.

#### *Experimental protocol*

Mycoplasma free, breast carcinoma cells were plated in 35 mm Petri dishes in DMEM supplemented with 5% heat-inactivated fetal bovine serum and stripped with 2.5% dextran-coated charcoal (FBS-S) to reduce the endogenous steroid concentrations. The medium was discarded 24 h after plating and cultures were treated with HF  $5 \times 10^{-6}$  M or  $1 \times 10^{-7}$  M DHT dissolved in ethanolic solution immediately before each experiment and added to growth medium. The final concentration of ethanol was <0.1%.

Since we found an increased cell proliferation when EVSA-T cultures were treated in media supplemented with DHT  $1 \times 10^{-7}$  M (Fig. 1), this quantity was used in experiments. The HF concentration was chosen according to dose-response curves (Fig. 2).

EVSA-T cells were divided into three aliquots: the first was used as a control (C) and in this portion AR were evaluated at time 0, 24, 48 and 72 h. The cells from the second portion were incubated with DHT ( $1 \times 10^{-7}$  M), and then the AR were assayed after 24, 48 and 72 h. In the third portion, the AR were evaluated after incubation with HF ( $5 \times 10^{-6}$  M) at time 24 and 48 h, and 24 and 48 h after washing for the removal of HF from the cells. Moreover, the cells treated with HF at time 24 and 48 h were washed and then incubated with DHT ( $1 \times 10^{-7}$  M) and the AR were assayed after a 24 and 48 h incubation period.

#### *[ $^3\text{H}$ ]Thymidine incorporation*

EVSA-T cells were seeded in appropriate numbers in 35 mm Petri dishes and treated as mentioned above. [ $^3\text{H}$ ]Thymidine (2  $\mu\text{Ci}/\text{ml}$ ) was added 2 h before the end of each treatment. After incubation, the medium containing [ $^3\text{H}$ ]thymidine was aspirated, cells were washed twice with ice-cold PBS (phosphate buffered saline), solubilized with 1.5 ml warm SDS (sodium dodecyl sulfate) 0.1% and transferred to tubes with 1.5 ml ice-cold PCA (perchloric acid) 1 N. After 1 h incubation on ice, the tubes were centrifuged (800 g for 10 min at 4°C): the supernatant was aspirated and the pellets were resuspended in 2 ml ice-cold PCA 0.5 N and centrifuged as described above. The pellets were then resuspended in 0.5 ml ice-cold PCA 0.5 N, incubated in a shaker for 30 min at 70°C and centrifuged. 200  $\mu\text{l}$  of the supernatant was added to 2 ml of scintillation liquid and counted in a  $\beta$ -counter.

#### *Cell counting*

The cells were plated in 35 mm Petri dishes at a density of 50,000 cells/dish and maintained as described above. DHT and HF were added to the medium according to the experimental protocol and at various times triplicate dishes were counted in a hemocytometric chamber.

#### *AR assay*

The assay of ARs, both cytosolic and nuclear, was carried out by an exchange method previously described [18]. All procedures were carried out at 4°C, unless otherwise indicated. Cell concentrations ranging from  $5 \times 10^6$  to  $6 \times 10^6$  cells/ml were used in the experiments. Briefly, cells were homogenized in buffer A (10 mM Tris pH 7.4 at 20°C, 1.5 mM EDTA, 10% glycerol v/v) and pelleted by centrifugation at 800 g for

10 min. The supernatant was adjusted to contain 10 mM sodium molybdate, and then centrifuged at 100,000 *g* for 60 min to yield the soluble cytosol fraction. The nuclear pellet was treated with pancreatic DNase I (20  $\mu$ g/ml final concentration, Sigma) for 30 min and then centrifuged at 800 *g*. The pellet was incubated in KCl buffer (10 mM Tris pH 7.4 at 20°C, 1.5 mM EDTA, 10 mM thioglycerol, 0.6 M KCl, and 10% v/v glycerol), and centrifuged at 100,000 *g* for 30 min (KCl-extractable fraction). The residual pellets, after washing, were resuspended in buffer A. This fraction contained KCl-non-extractable nuclear receptors. In order to decrease the non-specific binding and to remove endogenous steroids, the cytosolic and KCl-extractable fractions were mixed for 1 h with buffer containing dextran-coated charcoal (DCC) 0.1% and then pelleted. Samples for the protein assays (Bradford's method [19] using bovine serum albumin as standard) were drawn from the supernatant after ultracentrifugation. The DNA contents were determined according to the method of Burton (using calf thymus DNA as standard) [20], before adding the DNase. Incubation of cytosol fraction, KCl-extractable fraction and KCl-non-extractable fraction was carried out for 2 h at 20°C in quadruplicate, using [<sup>3</sup>H]R 1881 at a final concentration of 5 nM and a 200-fold molar excess of triamcinolone acetonide. The non-saturable binding, measured by parallel incubation in the presence of a 200-fold molar excess of corresponding unlabeled R 1881, was subtracted from the total binding. The unbound hormone was removed by 0.25% DCC incubation for 30 min and centrifuged for 15 min. Aliquots of 400  $\mu$ l of supernatant were placed in vials containing 8 ml of scintillation liquid (Atomlight NEN Chemicals) and then counted in a Beckman LS 7000  $\beta$ -counter (Beckman Instruments Inc., CA, 55% efficiency). Results are expressed as fmol/mg protein for cytosolic receptors or fmol/mg DNA for nuclear receptors. The nuclear AR level is the sum of the levels in KCl-extractable and KCl-non-extractable fractions.

Scatchard's analysis [21] of saturation data was used to quantify maximal binding capacity and affinity parameters (dissociation constant,  $K_d$ ) by means of the Enzfitter computer program. The Scatchard analysis was performed on three samples of control cells, two samples of cells incubated with DHT for 24 h and two samples of cells incubated with HF for 24 h.

Aliquots of cytosol, KCl-extractable and KCl-non-extractable fraction were incubated with increasing concentrations (0.625–10 mM) of labeled R 1881, with and without an excess of unlabeled R 1881. The results obtained using the single point assay correlated with those using Scatchard's analysis; the concentration of ligand adopted (5 nM) provided a reliable, although underestimated, measurement of AR content. The correlation of the single saturation point assay with the Scatchard analysis was  $r = 0.90$ .

#### Estrogen receptor (ER) assay

The assay was performed as described above. Determination of ER binding activity was made in cytosol, KCl-extractable and KCl-non-extractable fraction after incubation with 5 nM [<sup>3</sup>H]estradiol (96 Ci/mmol), Amersham Int. Lab. (England) with or without 1  $\mu$ M diethylstilbestrol for 4 h at 20°C.

## RESULTS

We tested the effect of androgen (Fig. 1) on cell proliferation by treating cells for 24, 48 and 72 h with increased DHT concentrations and found the best stimulation of cell proliferation at concentrations  $10^{-7}$  M. While the concentrations  $10^{-10}$  and  $10^{-9}$  M did not produce substantial variations in cell growth, the  $10^{-5}$  M concentrations showed a 25% inhibition at 48 and 72 h followed by partial recovery after 96 h. This effect is also present after a 7-day treatment (data not shown). The inhibitory effect of HF on cell growth is illustrated in Fig. 2, after 7 days

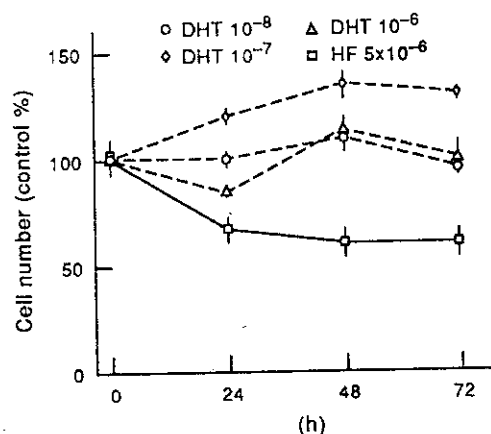


Fig. 1. Dose dependence of cell growth by DHT at increasing molar concentrations and effect of the antiandrogen HF in EVSA-T cells. Data represent the mean  $\pm$  SEM of three separate experiments, each performed with triplicate cultures.

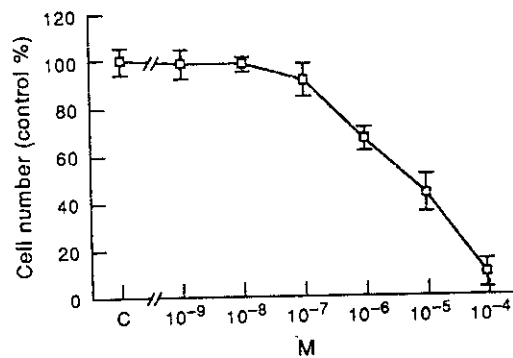


Fig. 2. Effect of increasing concentrations of HF on EVSA-T proliferation after 7 days of antiandrogen exposure. The data are expressed as the mean  $\pm$  SEM of triplicate cultures.

of incubation at increasing doses. Figure 1 shows the effect on EVSA-T cells at the chosen dose and during experimental times.

Our results show that EVSA-T cells have cytosolic and nuclear ARs (Table 1). By the Scatchard analysis (Table 2), the specific high affinity binding is demonstrable.

The AR content is not modified by the experimental times and is not affected by culture medium if heat-inactivated FBS-S with ethanolic vehicle alone is added (Table 1). Moreover, the washing and medium change after 24 and 48 h incubation had no effect on AR in comparison with basal levels. When DHT ( $1 \times 10^{-7}$  M) was added to the medium (FBS-S), we observed a great increase in nuclear AR levels after 24 h. This value remained constant even after 48 and 72 h of steroid incubation. The cytosolic receptor quantities showed a decrease after 24 h of DHT incubation and then reverted to control values (Table 1).

In HF-treated cells, nuclear AR increased after 24 h and remained at this level after 48 h. When the antiandrogen was removed by washing, the nuclear AR content became similar to that of control cells (Fig. 3).

In cultured cells treated with HF for 24 h, the addition of DHT to the medium increased the nuclear AR fraction, though to a lesser extent than that observed in cells treated with DHT alone. Similar results were obtained when DHT

Table 1. AR levels (cytosolic: ARc, fmol/mg protein; nuclear: ARn, fmol/mg DNA; mean  $\pm$  SEM) in EVSA-T cells

Time of incubation (h)	0	24	48	72
Control cells				
ARc	12 $\pm$ 1.4	18 $\pm$ 0.6	16 $\pm$ 1.4	18 $\pm$ 1.4
ARn	52 $\pm$ 3.3	65 $\pm$ 4.8	51 $\pm$ 2.0	58 $\pm$ 2.1
DHT treatment				
ARc		3 $\pm$ 0.4	12 $\pm$ 0.9	15 $\pm$ 1.8
ARn		162 $\pm$ 6.2	173 $\pm$ 2.2	169 $\pm$ 7.9
HF treatment				
ARc		4 $\pm$ 0.5	13 $\pm$ 1.7	
ARn		95 $\pm$ 2.2	96 $\pm$ 1.4	

Values are means of quadruplicate experiments.

was added to medium after 48 h of pretreatment with HF (Fig. 3).

Effects of DHT and HF on cell growth are shown in Fig. 4. The population-doubling time of EVSA-T cells was 45 h. When  $1 \times 10^{-7}$  M DHT was added to FBS-S supplemented medium, the proliferation rate of cells was faster than that of control cells. DHT stimulated cell growth after 24 h (+20%) and 48 h (+35%) while the antiandrogen HF had an inhibitory effect (-40%) in comparison to control cells, both after 24 and 48 h of treatment. It is interesting that DHT incubation after pretreatment with HF for 24 or 48 h was unable to restore cell growth even to the values of the control cultures. Our results concerning cell growth are in agreement with data relating to the incorporation of thymidine into the cells during experiments. Indeed, incorporation of thymidine increased by 21% after 24 h and by 35% after 48 h compared with control levels at a DHT concentration of  $1 \times 10^{-7}$  M. When HF was added to medium, [<sup>3</sup>H]thymidine incorporation decreased by 35% after 24 h of incubation and thereafter remained stable (Fig. 5).

When the medium containing HF was replaced with DMEM-FBS-S medium, the levels of thymidine incorporation fell further below that of HF-treated cells. Not even treatment with DHT was able to restore the incorporation of thymidine to former levels; on the contrary, lower values were registered than for cells treated with antiandrogen (Fig. 6). It is noteworthy that, after treatment with HF, no

Table 2. AR levels tested by Scatchard analysis in 3 samples of control cells, 2 samples of DHT-treated cells and 2 samples of HF-treated cells

Treatment	ARc	$K_d$ (nM)	ARn		ARn	
			KCl-extr.	$K_d$ (nM)	KCl non-extr.	$K_d$ (nM)
Control	18 $\pm$ 2.4	0.8 $\pm$ 0.1	51 $\pm$ 4.2	0.8 $\pm$ 0.2	10 $\pm$ 3.1	0.85 $\pm$ 0.1
DHT	5.4 $\pm$ 3	0.7 $\pm$ 0.05	134 $\pm$ 6.8	0.6 $\pm$ 0.1	35 $\pm$ 3.5	0.6 $\pm$ 0.07
HF	3.3 $\pm$ 2	1.1 $\pm$ 0.1	76 $\pm$ 5.9	0.8 $\pm$ 0.1	23 $\pm$ 2.5	0.8 $\pm$ 0.1

Data are expressed as mean  $\pm$  SD; ARc: cytosolic AR, fmol/mg protein; ARn: nuclear AR, fmol/mg DNA, assayed on KCl-extractable fraction and KCl-non-extractable fraction.

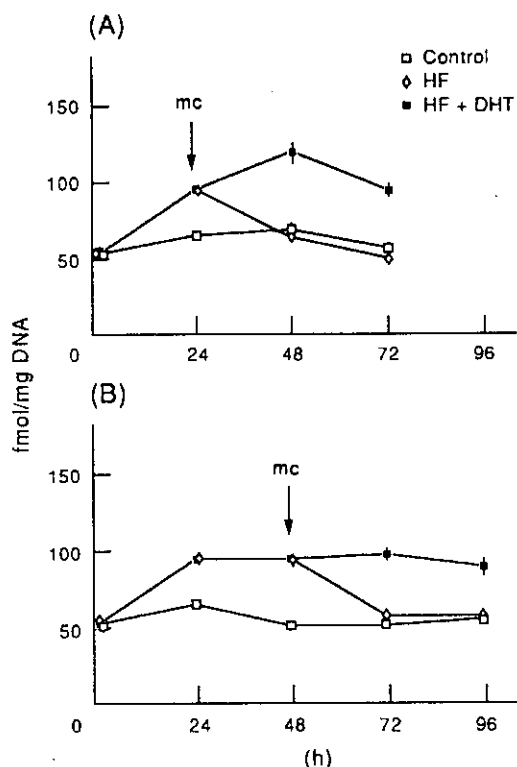


Fig. 3. Nuclear AR levels in cells incubated with medium supplemented with FBS-S (control) alone, or HF for 24 h (panel A) or 48 h (panel B). Subsequently the medium was changed (mc) with FBS-S medium in control and HF-treated cells or HF-treated cells were incubated with DHT  $1 \times 10^{-7}$  M (HF + DHT). Data represent the mean  $\pm$  SEM of four separate experiments.

degenerative phenomena were observed in cell cultures.

To discriminate a possible action of DHT and HF through ER, we investigated their effects on ER levels in triplicate cultures during two different experiments. In control cells we found low ER content in the cytosol fraction ( $<4$  fmol/mg protein), while nuclear ER levels were  $35 \pm 8$  fmol/mg DNA (mean  $\pm$  SD). After incubation with DHT for 24 or 48 h, nuclear ER levels were unchanged (at 24 h:  $41 \pm 7$ , and at 48 h:  $34 \pm 5$  fmol/mg DNA, mean  $\pm$  SD). Likewise, the treatment with HF did not modify nuclear ER quantities found in control cells (at 24 h:  $30 \pm 9$ , and at 48 h:  $31 \pm 7$  fmol/mg DNA, mean  $\pm$  SD).

To further verify whether the action of DHT on cell growth is exerted by an ER-mediated mechanism, we studied the effect of antiestrogen tamoxifen (Tam) on thymidine incorporation during two different experiments. The treatment with Tam ( $1 \times 10^{-6}$  M) induced a decrease in thymidine incorporation ( $25 \pm 5\%$ , mean  $\pm$  SD, lower than control) after 24 h of incubation. This inhibition is constant (range 15–30%) and, at 96 h of treatment, the value is  $15 \pm 7\%$  (mean  $\pm$  SD) lower than that of control cells.

The simultaneous incubation of cells with Tam and HF decreases incorporation of

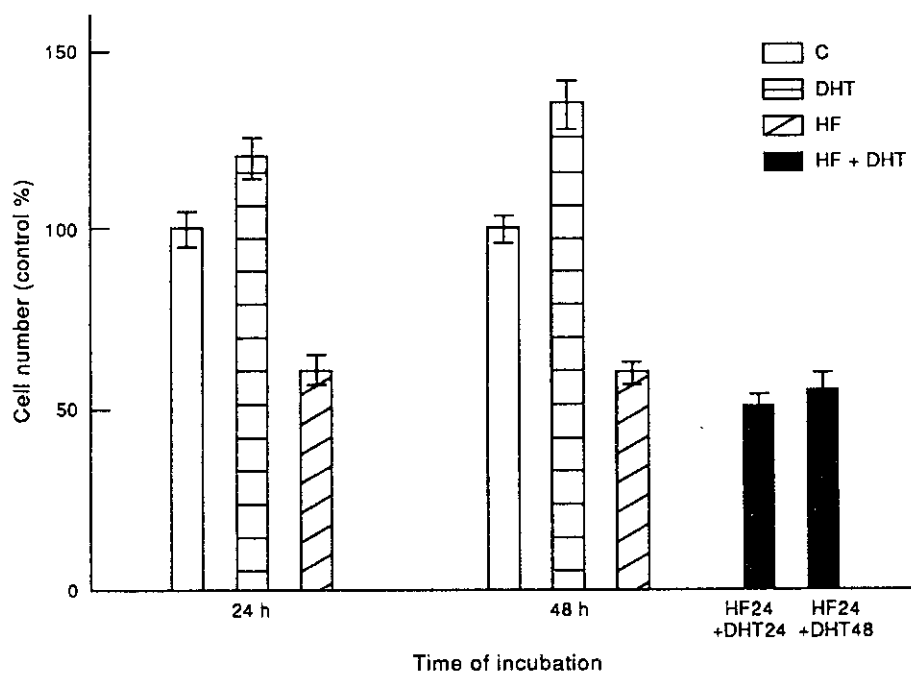


Fig. 4. Effect of DHT and HF on cell growth. Each bar represents the mean of triplicate culture plates. Results are expressed as the percentage variation ( $\pm$ SD) in final cell number compared with control plates at the same time of incubation. The two bars on the right indicate the proliferation of cells treated with HF ( $5 \times 10^{-6}$  M) for 24 h and subsequently with DHT ( $1 \times 10^{-7}$  M) for 24 or 48 h.

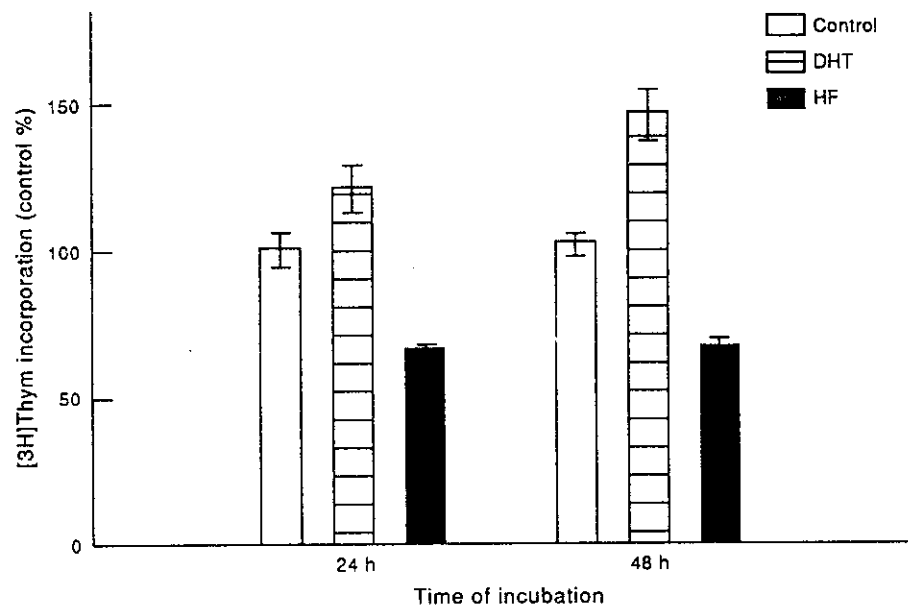


Fig. 5. Effects of DHT and HF on the incorporation of [<sup>3</sup>H]thymidine in EVSA-T cells. Values are means of quadruplicate experiments and results are expressed as percentages ( $\pm$ SEM) of control cells.

thymidine by  $55 \pm 6\%$  (mean  $\pm$  SD) in comparison with controls both at 24 and 48 h.

#### DISCUSSION

Our data show the stimulatory effect exerted *in vitro* by DHT on proliferation yields of EVSA-T carcinoma cell line. Since ARs are increased after incubation with DHT, in these cells the androgen action seems to be mediated by the AR pathway. In accordance with previous studies *in vivo* [14–16], after 24 h of steroid incubation, we found a decrease in cytosolic AR and a rise in nuclear AR of 180% compared with control cells. This increased level was maintained until the 72nd h of incubation.

However, there is a discrepancy between concentrations of DHT that half-maximally saturate AR and concentrations that half-maximally stimulate cellular growth [2]. This may mean that all receptor sites must be occupied by androgen before any stimulatory response on DNA synthesis is induced. Although several studies have demonstrated that  $10^{-7}$  M DHT stimulates breast cancer cell growth (such as MCF-7 cell line) by acting through ER [3], our report cannot confirm that this event occurs in EVSA-T cells. Indeed, ER levels were unchanged during incubation with DHT, as were nuclear progesterone receptors, an index of the estrogen stimulation pathway (data not shown).

Recent studies have demonstrated that androgens behave as estrogen agonists at

supraphysiological concentrations [2, 3], while physiological concentrations of androgens inhibit both basal and estrogen-induced cell proliferation in the estrogen-responsive ZR-75-1 breast cancer cell line through their interaction with the AR [5, 22]. In our experiments, EVSA-T cell line, which has never been reported as steroid-dependent, seems to be partially androgen-sensitive. Moreover, the DHT action does not appear to interact with ER proteins, and cell growth is inhibited by HF, an antiandrogen well known as having no significant affinity for receptors other than the AR [6–8]. Indeed, the antiproliferative activity of Tam in this cell line seems to be additive to that exerted by antiandrogen HF, and could be due to the nuclear ER amount found in these cells.

As shown in Table 1, HF treatment of cells leads to an increase in nuclear AR content, though this effect is lower than that induced by DHT. This finding is in line with data from *in vivo* studies on the effect of HF [16]. Our results suggest that HF does not act by blocking the translocation of antiandrogen–receptor complexes [7, 13, 14] but by increasing their nuclear accumulation. HF-induced AR–antiandrogen complexes are biologically inactive, as evidenced *in vivo* [16] by the inhibition of androgen-regulated proteins.

It is difficult to explain the fact that the addition of FBS-S medium or DHT to cells pretreated with HF changes the nuclear AR content but does not reverse the effect of HF on

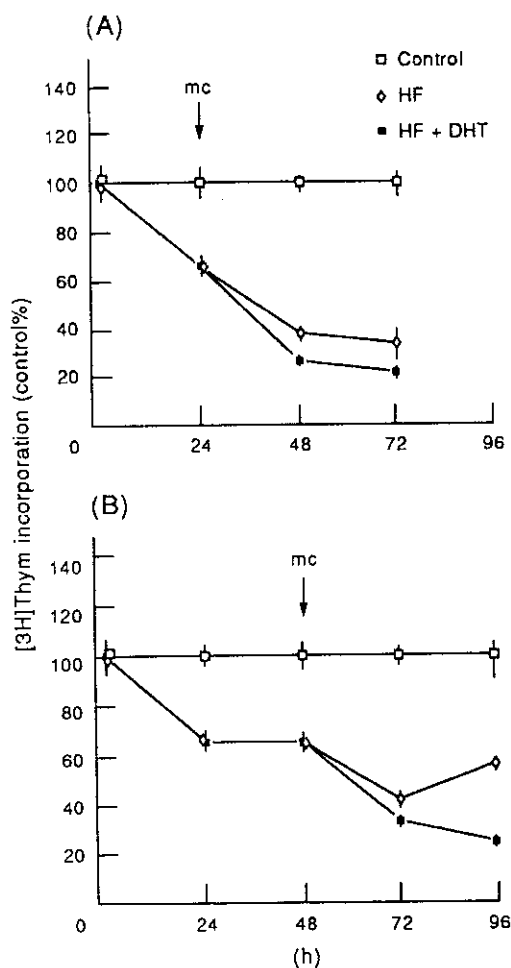


Fig. 6. Effect of medium change alone (mc) and medium change plus  $10^{-7}$  M DHT (HF + DHT) at 24 (panel A) and 48 h (panel B) on  $[^3\text{H}]$ thymidine incorporation in HF-treated EVSA-T cells (HF). FBS-S medium was also changed in control cells (control) at 24 or 48 h without addition of hormone. Values are expressed as percentages ( $\pm$  SEM) of control cells at the same time of incubation and represent the mean of four separate experiments.

cell growth. Considering that AR values after washing and medium change are similar to those of control cells (Fig. 6), the inhibition of growth may be caused by the depletion of growth promoting factors in heat-inactivated and charcoal-stripped medium.

The addition of DHT to HF-treated cells induces a greater block of thymidine incorporation than that evidenced during HF treatment. Although the AR content in cells is higher than that in HF-treated cells, the inhibition of thymidine incorporation is maintained even after 48 h of incubation (Fig. 6). A possible explanation may be that the steroid has to occupy all receptor sites in order to induce the stimulatory response on cell growth [2]. An alternative hypothesis is that the cells may

metabolize DHT to inactive steroid, as demonstrated in MCF-7 cells treated with DHT [2]. In this way, small changes in incubation conditions or cell density could drastically alter amounts of active androgen available to cells [2].

However, the lack of cell growth after medium change and addition of DHT to HF-treated cells could be due to the antiandrogen itself. It is possible either that the retention of high HF levels persists in cells for 2 days (i.e. a prolonged half-life of HF is demonstrated *in vivo* [23]) or that the protein synthesis of the cells is so depressed that they need more than 2 days after medium change to recover their proliferation.

In conclusion, although this cell line responds to DHT with an increased cell proliferation and to pure antiandrogen HF with inhibition of growth, it is possible that the effect of HF could be non-specific or that more complex interactions may explain the lack of growth after treatment with HF.

The present work demonstrates that EVSA-T cells are androgen-sensitive. Further studies are needed to clarify the interactions of hormones and antihormones with steroid receptors and to define the relationship between AR content and metabolic potency of androgen on cell growth in human breast cancers, *in vivo* and *in vitro*, and thus to estimate the response of tumors to hormonal and antihormonal manipulation.

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