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RU486, A Progestin and Glucocorticoid Antagonist, Inhibits the Growth of Breast Cancer Cells via the Progesterone Receptor*

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ABSTRACT. The progestin and glucocorticoid antagonist RU486 was tested on the growth of several cell lines in culture. RU486 inhibited the growth of two progesterone receptor (RP) positive human breast cancer cell lines (MCF7 and T47D). The antiproliferative effect was dose dependent and its magnitude correlated with the RP content of the tested cells (T47D > estradiol-primed MCF7 > withdrawn MCF7). Cell growth inhibition was not prevented by the addition of dexamethasone, dihydrotestosterone, or estradiol, but the cells were rescued by low concentrations of the progestin R5020. RU486 had no effect on the growth of two RP negative human breast cancer cell lines and a rat fibroblast cell line. Moreover, RU486 had no progestin agonist activity in T47D cells when evaluated by measuring the ^{35}S-labeling of two progestin-regulated proteins with mol wts of 48,000 and 250,000, but it totally prevented the induction of these two proteins by R5020. In conclusion, RU486 selectively inhibited the growth of human breast cancer cell lines with unoccupied RP sites and its effect was correlated with the RP concentration of these cells. We propose that RU486 is a RP-targeted drug of potential utility in breast cancer treatment. (*J Clin Endocrinol Metab* **50:** 692, 1985)

THE GROWTH of hormone-dependent breast cancer is specifically inhibited by synthetic antiestrogens via a mechanism which is thought to be mediated by the estrogen receptor (1–3) and to involve inhibition of the stimulatory effect of estrogen. Progestins and some androgens also are used in the endocrine therapy of breast cancer, where their effect is apparently mediated by their own receptors (4–7). However, high concentrations of these hormones are required, which leads to undesirable side effects. More potent and specific drugs are obviously required.

Recently, RU486 was found to be a potent antiglucocorticoid and antiprogestin (8–10). This compound binds with high affinity to the progesterone and glucocorticoid receptors, inhibits egg implantation, and has been proposed as a contraceptive agent (11).

In this study, we determined the effect of RU486 on the growth of several breast cancer cell lines which are progesterone receptor (RP) positive (MCF7, T47D) or negative (BT20, MDA-MB231). We successively addressed the following two questions: 1. Does RU486 affect cell growth? 2. How is this effect mediated within the cell, or more precisely, is it mediated by the glucocorticoid receptor, the RP, or other cellular structures?

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Materials and Methods

Steroids

RU486, 17β-hydroxy-11β-(4-methylaminophenyl)-17α-(1-propynyl) estra-4,9-dien-3 one-6-7, was provided by the Centre de Recherche Roussel-Uclaf (Sakiz, E., D. Philibert, Romainville, France). Estradiol (E_2), 5α-dihydrotestosterone (DHT), 9 α fluoro-16-methyl-prednisolone [dexamethasone (Dex)], and 17,21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione [promegestone (R5020)] were gifts from Roussel-Uclaf. All steroids were 98% pure as verified by thin layer chromatography.

Cell Culture

Clone 11 subline of the T47D breast cancer cell line (12) was donated by I. Keydar, Tel Aviv University (Tel Aviv, Israel). MCF7 breast cancer cells (13) were obtained from the Michigan Cancer Foundation (MT) and M. Lippman (National Cancer Institute, Bethesda, MD). BT20 (14) and MDA-MB231 (15) breast cancer cells were donated by V. Piczak (Mason Research Institute, Rockville, MD). The fibroblastic clone 49F of the normal rat-kidney cell line NRK (16) was obtained from P. Vigier (Institut du Radium, Orsay, France). T47D and BT20

cells were maintained in RPMI 1640 medium (Flow Laboratory, SA, Ayrshire, UK). This medium was supplemented with 10% fetal calf serum (FCS) and 0.6 μg/ml bovine insulin for T47D cells and with 15% FCS and 2 μg/ml bovine insulin for BT20 cells. MCF7 and MDA-MB231 cells were cultured in Dulbecco's modified Eagle's medium containing 10% FCS and 0.6 μg/ml insulin. 49F cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% FCS. Cells growing in monolayers were kept at 37 C in a humid atmosphere in the presence of 5% CO_2.

Steroid treatment of cells

Before each growth experiment, the cells were withdrawn from endogenous steroids for 6–7 days. They were first grown for 2 days in 5% or 10% dextran-coated charcoal treated serum (FCS/DCC) (17) and then in 3% FCS/DCC for 3 days (withdrawal medium). The cells were trypsinized and transferred to appropriate culture dishes to test the effects of hormones on DNA accumulation or protein synthesis in insulin-free withdrawal medium with a concentration of 1% or 3% FCS/DCC as specified in the figure legends. One or 2 days after plating, steroids were added to the culture medium in ethanolic solution with a final ethanol concentration of less than or equal to 0.1%. An equivalent volume of solvent was added to the control cells.

Cell growth assays

Cells were plated in wells 16 mm in diameter (24-well dishes, Linbro-Flow Laboratory). After hormone treatment, the amount of cellular DNA was evaluated *in situ* by diaminobenzoic acid fluorescence assay (18) on cells fixed with methanol. Alternatively the cell number was counted in sister wells on trypsinized cells using a Thoma hemocytometer.

Analysis of labeled proteins

Cells were plated in 100 μl medium containing 3% FCS/DCC in microwells 0.8 cm in diameter (96 μ-well dishes, Nunclon Delk, Roskilde, Denmark). After steroid stimulation, the cells were labeled with 200 μCi/ml L[^{35}S]methionine (Radiochemical Center, Amersham, England SA < 1000 Ci/mmol) in 60 μl Eagle's minimum essential medium containing one tenth the normal concentration of methionine for 6 h (released proteins) or 4 h (cellular proteins). Culture media were centrifuged at 700 × *g* for 5 min and/or at 9000 × *g* for 2 min. Cells rinsed twice with PBS were dissolved in 15 μl lysis buffer A (19, 20) containing 0.5% sodium dodecyl sulfate (SDS) plus 40 μl H_2O and lysed by three freeze-thaw cycles (−80 C).

Total incorporation of [^{35}S]methionine into proteins was evaluated by precipitation of 10 μl medium with 15% trichloroacetic acid (TCA) on Whatman 3MM filters as previously described (4). ^{35}S-Labeled proteins were analyzed by one-dimensional SDS-polyacrylamide gel electrophoresis. Gels then were processed for fluorography and exposed for 7–10 days at −80 C to Kodak X-omat S films [preflashed to an absorbance of 0.2 od as described (21)]. Films were scanned using a Vernon scanning densitometer, and the amount of specific protein was estimated as described (20).

Results

RU486 inhibited the growth of T47D and MCF7 cells, two RP positive breast cancer cell lines

T47D cell (clone 11) are responsive to E_2 (22), antiestrogen, and progestins (4). When cultured with 1% or 3% FCS/DCC without added estrogen, their growth was strongly inhibited by the progestin inhibitor RU486 (Fig. 1) and also by R5020, confirming a previous report (4). Based on cell number counts (Fig. 2) or cellular DNA measurements after 10 days treatment, dose-response

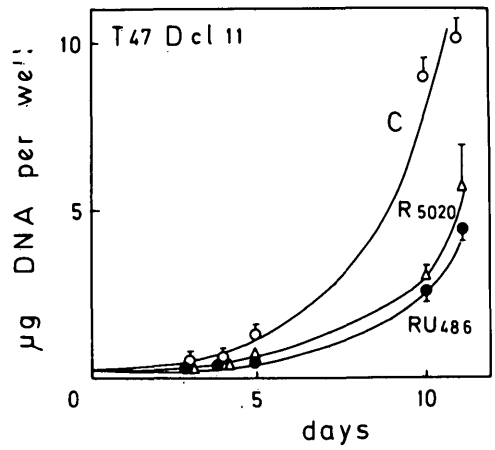

FIG. 1. Inhibition of the growth of T47D clone 11 breast cancer cells by RU486 and R5020. T47D cells grown for 5 days in RPMI containing FCS/DCC were plated in 24-well dishes at a density of 30,000 cells per well in medium containing 3% FCS/DCC without added insulin. After 1 day, attached cells were treated with RU486 (100 nM; ●), R5020 (100 nM; Δ), or solvent alone (C; ○) in medium containing 1% FCS/DCC. Culture media were changed every 2 days. Three wells from each series were fixed with methanol at different times and later assayed for DNA (see *Materials and Methods)*). Results are the mean ± SD of three separate determinations.

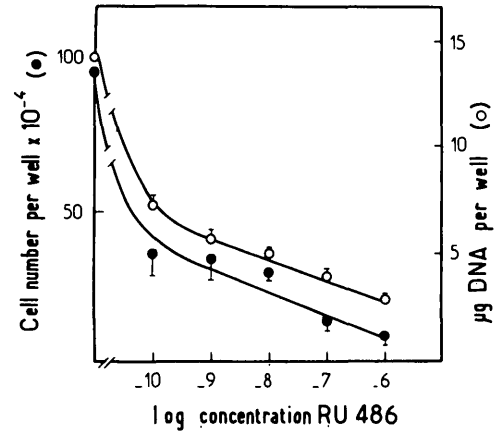

FIG. 2. Comparative effects of RU486 by cell number evaluation or DNA assay. T47D cells were plated as in Fig. 1. They then were treated with increasing concentrations of RU486. After 12 days, cells were trypsinized and counted with an hemocytometer. Values are the mean ± SD for three wells per point. In the same experiment, DNA was assayed in parallel triplicate wells.

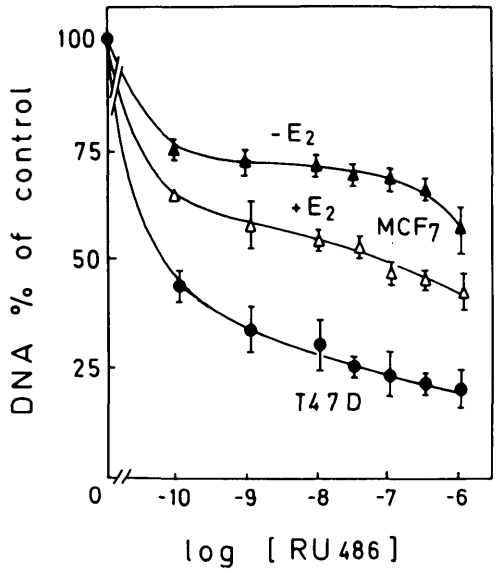

FIG. 3. Effect of RU486 on the growth of T47D and MCF7 cells: dose-response curve. MCF7 cells were grown for 7 days in medium containing 10% FCS/DCC. They then were trypsinized and cultured for 5 days in medium containing charcoal-stripped serum (10% FCS/DCC) with (△) or without (▲) 10 nM E_2. T47D cells (●) were grown for 5 days as described in Fig. 1. Cells then were plated in 24-well dishes at a density of 20,000 cells per well in medium containing 3% FCS/DCC without insulin. Two days later, cells were treated with increasing concentrations of RU486 for 10 days, and they were fixed *in situ* and processed for DNA assay. Results are expressed as percentages of control DNA. The absolute values of control DNA were 17 ± 0.5 (SD) μg, respectively, for untreated MCF7 cells, 11.4 ± 2 μg for E_2-pretreated MCF7 cells, and 12.5 ± 0.4 μg for T47D cells.

curves demonstrated a concentration-dependent cell growth inhibition. A suboptimal effect was obtained with a 1 nM concentration of RU486, consistent with an effect mediated by the RP, the glucocorticoid receptor, or both. Inhibition by RU486 was also found in MCF7 cells (Fig. 3), but it was weaker than in T47D cells. When MCF7 cells were pretreated for 9 days with E_2 to increase the RP level (23), RU486 was more efficient than without pretreatment, suggesting a correlation between RP concentration and RU486 efficacy. The continuous presence of E_2 did not prevent the inhibitory effect of RU486 (data not shown).

Growth inhibition by RU486 was prevented by the occupation of the RP, and not by that of other steroid receptors

RU486 binds to several classes of receptors: glucocorticoid, progesterone, and androgen (8). To determine the nature of the receptor involved in the antiproliferative activity of RU486, we first tried to rescue the cells by occupying the different receptors by their corresponding high affinity ligands. Figure 4b shows that in T47D cells, Dex, and DHT were unable to prevent growth inhibition by RU486. However, they were themselves inhibitory at the concentration required to prevent the binding of

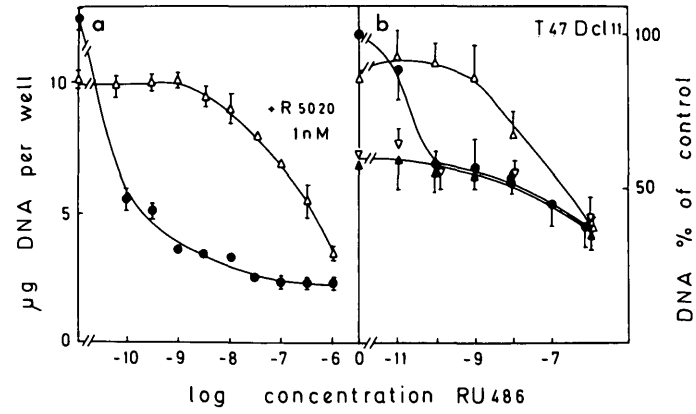

FIG. 4. Effect of RU486 on the growth of T47D cells in the presence of other steroids. a, T47D cells were grown and plated as in Fig. 1. They then were treated for 14 days with increasing concentrations of RU486 alone (●) or of RU486 + 1 nM R5020 (△). b, T47D cells were treated for 10 days with increasing concentrations of RU486 alone (●) or with RU486 + 100 nM Dex (▲) or RU486 + 100 nM DHT (▽) or RU486 + 1 nM R5020 (△). Total cellular DNA was assayed on triplicate wells. Results are expressed as micrograms of DNA per well (a) or as percentages of the amount of DNA in untreated control cells (b) which were 8.7 ± 0.4 (SD) μg (●), 6.90 ± 0.42 μg (△), 5.73 ± 0.40 μg (▽) and 5 ± 0.7 μg (▲), respectively.

RU486. In contast, a low concentration (1 nM) of R5020 protected the cells against the antimitogenic effect of RU486 (Fig. 4a). Since higher concentrations of R5020 are also growth inhibitory (4), the cells were not totally rescued from the effect of higher concentrations of RU486. However, the relative efficacy of several concentrations of R5020 in preventing growth inhibition by RU486 is clearly compatible with the relative affinities of the two ligands for the RP (9) (Fig. 4). Since only R5020 was able to protect the cells from the inhibitory effect of RU486, we conclude that this effect was probably mediated by the RP.

RU486 did not inhibit the growth of RP negative cell lines

In order to assess the specificity of this inhibition, we tested other cell lines which are known to be unresponsive to estrogen and progesterone. Figure 5 shows that RU486 was totally ineffective in two RP negative breast cancer cell lines and in one fibroblast cell line which was treated as RP positive cell lines. In MDA-MB231 cells, RU486 was not effective either alone or associated with DHT (Fig. 5a). In the BT20 RP negative breast cancer cell line, RU486 and R5020 had no significant effect when tested separately (Fig. 5b). In the mouse fibroblast 49F, Dex is a growth inhibitor, as in other fibroblasts (24) (Fig. 5c). RU486 alone or with DHT had no significant effect; however, at high concentrations, RU486 slightly prevented growth inhibition by Dex, which is in agreement with a previous report (25).

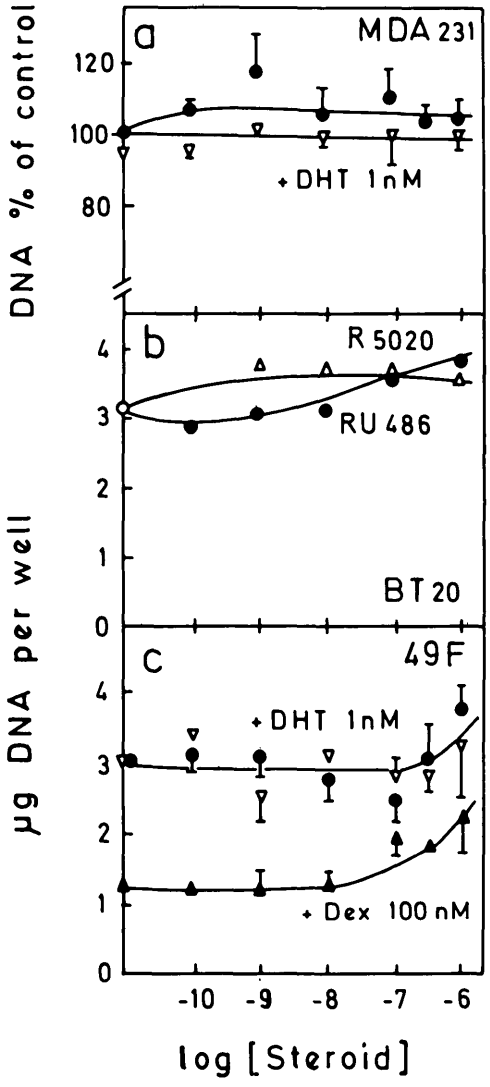

FIG. 5. Absence of cell-growth inhibition by RU486 in RP negative cell lines. The three indicated cell lines were treated with increasing concentrations of RU486 for 10 days (●), as in Fig. 2. The addition of androgen, progestin, or glucocorticoid was tested as follows: a, MDA MB231 cells also treated with 1 nM DHT (▽). b, BT20 cells also treated with R5020 alone (△). c, 49F fibroblast cells also treated with 1 nM DHT (▽) and with 100 nM Dex (▲). DNA was assayed on triplicate wells. Results are expressed as micrograms of DNA per well (b, c) or as a percentage of control DNA (a), which was 7.45 ± 0.5 (SD) μg.

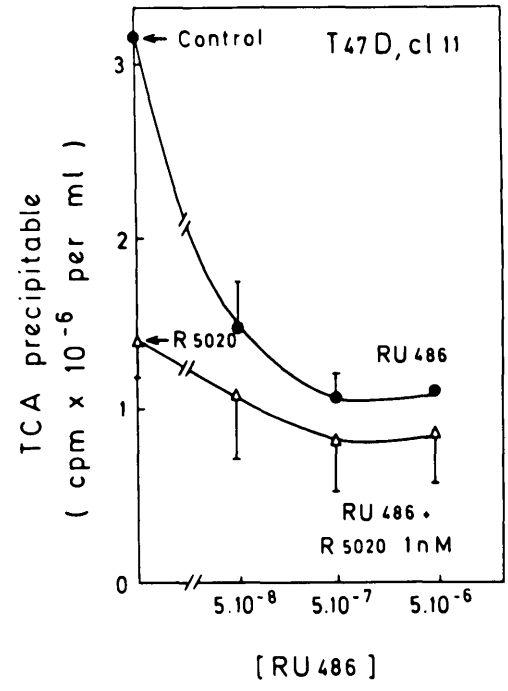

FIG. 6. Effect of RU486 on total proteins released in the medium. T47D cells were plated at a density of 12,000 cells per microwell in a medium containing 3% FCS/DCC. After 2 days, cells were treated for 5 days with RU486 alone (●) or in the presence of R5020 (△). Cells were then labeled with [^{35}S]methionine (see *Materials and Methods*). The total incorporation of ^{35}S-methionine into the proteins of conditioned media was evaluated by TCA precipitation as described in *Materials and Methods*. Results are expressed in counts per min of TCA-precipitable material/ml conditioned media.

Effect of RU486 on protein synthesis and release

In the T47D cell line, RU486 as well as R5020 (4, 26) markedly inhibited the production of proteins in the culture medium, as determined by labeling the proteins with [^{35}S]methionine and evaluating TCA-precipitable material. Figure 6 shows that in the absence of R5020 (*upper curve*), the degree of inhibition was more marked than in the presence of R5020. This is consistent with the inherent inhibitory effect of R5020 on the production of proteins in the medium (4, 26). However, the effects of RU486 and R5020 were not additive and it is possible that R5020 inhibits the efficiency of RU486 by preventing its binding to RP as in Fig. 4. We then studied the effect of RU486 on two progesterone-regulated proteins in T47D cells: the 48,000 mol wt protein secreted in the medium (26) and the cellular 250,000 mol wt protein (20). Figure 7 shows the specific effect of one concentration of R5020 (1 nM) and RU486 (10 nM). The labeling of the secreted 48,000 mol wt and of the cellular 250,000 mol wt proteins were both increased by R5020. RU486 was totally inactive in stimulating the production of these two proteins (*lanes 2 and 6*) but totally prevented their induction by R5020 (*lanes 4 and 8*). The relative amounts of the two proteins were estimated by scanning the fluorogram, as previously described (26). Figure 8 shows the effect of increasing the concentrations of both ligands. The two proteins were increased in a dose-dependent manner by R5020, as already reported (26), whereas RU486 alone had no effect at any concentration on the production of these two progestin-regulated proteins. This result confirms previous reports in which RU486 inhibited the effect of R5020 on the production of progestin- and androgen-regulated proteins (26), and clearly indicates that in this system the drug has no

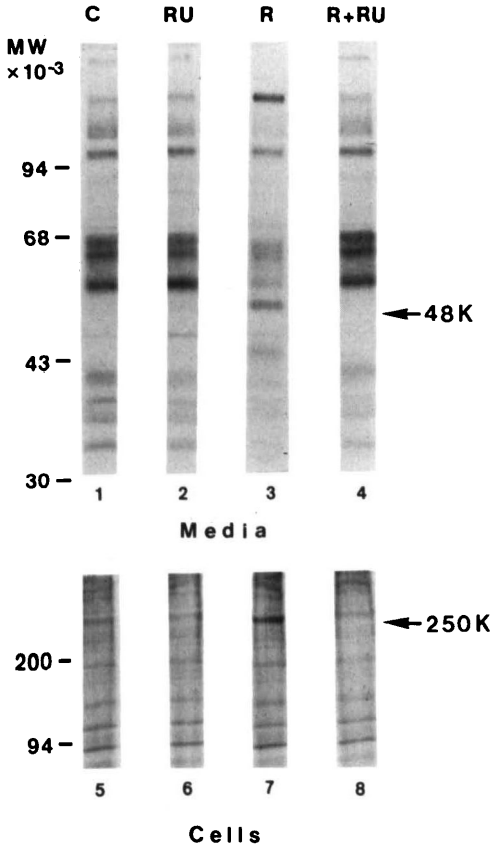

FIG. 7. Effect of RU486 on two progestin-specific proteins: SDS polyacrylamide gel. T47D cells (clone 11) were withdrawn from steroids and plated in microwells (20,000 cells per wells) as described in Fig. 1. Two days later, they were incubated for 4 days either with vehicle (C, 1, 5), 10 nM RU486 (RU, 2, 6), 1 nM R5020 (R, 3, 7), or 10 nM RU486 plus 1 nM R5020 (R + RU, 4, 8). The cells were labeled with [^{35}S] methionine as described in *Materials and Methods*. The same amount of TCA-precipitable [^{35}S]methionine labeled protein released into the media [5,000 cpm (*lanes* 1 to 4)] and present in the cells (150,000 cpm (*lanes* 5 to 8)] was analyzed on SDS 12% polyacrylamide gels. Labeled proteins were revealed by fluorography as described in *Materials and Methods*. Migrations of standard proteins are indicated on the *left*.

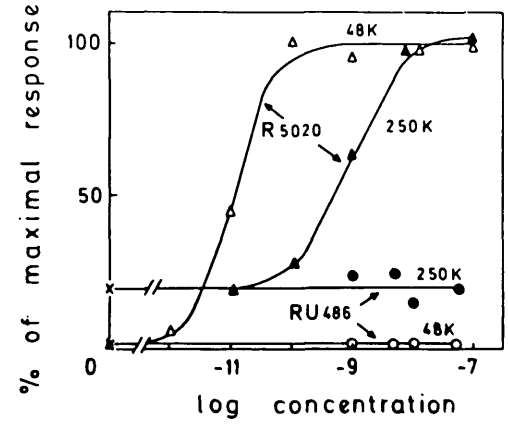

FIG. 8. Effect of RU486 on two progestin-specific proteins: dose response curves. T47D cells were incubated with vehicle (×) or the indicated concentrations of R5020 (△, ▲) or RU486 (○, ●). After [^{35}S] methionine incorporation, proteins released into the media and cellular proteins were analyzed on 12% SDS polyacrylamide gels as described in Fig. 6 and *Materials and Methods*. The amounts of cellular 250 K protein (▲, ●) and of released 48 K protein (○, △) were estimated as described in *Materials and Methods* and expressed as percentages of the maximal response obtained with R5020.

progestin activity and behaves as a full progestin antagonist.

Discussion

RU486 is a synthetic steroid bearing a dimethylaminophenyl ring at position 11β resembling the third phenyl ring of synthetic antiestrogen (10). Its potent antagonist activity is related to its high affinity for RP and glucocorticoid receptors and its absence of agonist activity.

In addition to its antagonist properties, previously described both *in vivo* (8, 9, 11) and *in vitro* (10, 25, 27, 28) we found that at low concentrations this drug inhibits the growth in culture of two hormone-responsive breast cancer cell lines but not that of nonresponsive cell lines. Several results strongly suggest that the antiproliferative effect of RU486 requires the presence of available RP sites. The antiproliferative effect was found only in RP positive lines and the extent of the effect was correlated to RP concentration. The RP positive cells were rescued from RU486 inhibition only by the progestin R5020, and not by a glucocorticoid, an androgen, or E_2. In the case of nonsteroidal antiestrogens, the degree of activation of the estrogen receptor can be evoked to explain the opposing effects of estrogens, which fully activate the receptor and stimulate growth, and of antiestrogens which activate the receptor less efficiently and inhibit cell growth (29, 30). By contrast the progestin antagonist RU486 has apparently no progestin activity since it did not stimulate the production of the 48,000 mol wt (26) and 250,000 mol wt (20) proteins in T47D breast cancer cells, nor does it induce uteroglobin in the rabbit uterus (E. Milgrom, personal communication). However, it can, like the progestin agonist R5020, inhibit the growth of RP positive cell lines *in vitro*. This suggests that the drug itself may inhibit cell growth as a cytotoxic agent rather than by preventing RP activation. However, since RU486 is inactive in RP negative cells, it acts specifically as a receptor-directed cytotoxic agent.

The decreased production of glycoproteins released by the cells (which are putative growth factors) may be a common intermediary pathway by which antiestrogens and RU486 inhibit cell proliferation (2, 4). In fact, it has recently been shown that such glycoproteins released by MCF7 cells stimulated the growth of resting cells (31).

On the basis of the low concentrations of RU486 required, and of the specificity of its action on RP positive cells, RU486 appears to be a potentially attrac-

tive drug for use in the treatment of certain breast cancers. However, its strong antiglucocorticoid activity may limit its therapeutic potential, even though it has recently been found that a reduced dosage may decrease the consequences of this antiglucocorticoid activity (32). A drug from the same series, whose antiglucocorticoid activity has been totally eliminated, would certainly be more attractive.

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