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Effects of Progesterone and R2323 on the Development of Dimethylbenzanthracene-induced Mammary Tumors

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Abstract *When started the day following DMBA administration, daily treatment with* 0.5 *or* 4 mg *of progesterone increased the early incidence and number of mammary tumors although maximal values measured* 141 days *after DMBA administration were similar to controls. At a daily dose of* 0.5 mg, *progesterone caused a marked stimulation in the size of the tumors. The lower dose of progesterone slightly increased estradiol binding while the higher dose reduced R5020 binding to tumors removed after sacrifice* (day 141). *Prolactin receptor levels in the tumors and plasma prolactin levels were unaffected by progesterone treatment whereas plasma LH levels were significantly reduced. Daily aaministration of increasing doses of R2323, a compound with antiprogestin activity, caused a progressive inhibition of tumor development. There was no significant change of receptor levels for estradiol, progesterone or prolactin in tumor tissue nor of plasma prolactin concentrations, whereas plasma LH levels were markedly decreased after R2323 treatment. This inhibitory effect on gonadotropin secretion could be mainly responsible for the potent inhibition of tumor development and growth accompanying treatment with the synthetic steroid. However, it remains possible that part of the inhibitory action of R2323 may be exerted directly at the tumor level.*

INTRODUCTION

MAMMARY tumors induced in the rat by 7,12-dimethylbenz(a)anthracene (DMBA) are well known to be dependent upon both estrogens and prolactin for development and growth [1–6]. The role of progesterone in the growth of these tumors is however less well characterized. Progesterone stimulated tumor growth when given after DMBA administration [7, 8] whereas when given to rats for a few weeks before and after DMBA administration, it was found to cause a reduction in the incidence, number and size of tumors [9, 10].

An investigation of the role of progesterone in the control of these tumors is of special interest in view of our report identifying the presence and characteristics of a specific progesterone receptor in DMBA tumors [11]. Since estrogens stimulate the level of progesterone receptors in the uterus [12, 13], it has in fact been proposed that the presence of a progesterone receptor in the tumor would be evidence that part of the normal hormonal control system of estrogens is functional [14]. Moreover, we have recently found that estradiol also induces increased levels of progesterone receptors in DMBA tumors [15].

It was thus felt of interest to study the effect of progesterone and R2323 (13α-ethyl-17-hydroxy-18,19-dinor-17β-pregna-4,9,11-triene-20yn-3-one), a compound with antiprogestin activity [16–18], on the development of mammary carcinoma induced by DMBA. In an attempt to correlate the response of these treatments with hormone receptor levels, the concentration of receptors for 17β-estradiol, progesterone and prolactin was measured in individual tumors.

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MATERIALS AND METHODS

Treatments

Female Sprague–Dawley rats (obtained

from Canadian Breeding Farms, St. Constant, Quebec) were injected intravenously with 5 mg DMBA at 50–55 days of age. The emulsion was kindly provided by Dr. Paul Schurr, The Upjohn Company, Kalamazoo, Michigan. Animals were housed 2 per cage with a lighting regimen of 14 hr light–10 hr darkness (lights on between 05:00 and 19:00 hr) and received Purina rat chow and water *ad libitum*.

From the day after DMBA was administered, the animals received daily subcutaneous injections of progesterone at doses of 0.5 or 4 mg or the vehicle alone (0.2 ml of 1% gelatin–0.9% NaCl). In a separate experiment, animals were injected daily with R2323 at doses of 2.5, 10, 30, 70 or 150 μg or with the vehicle alone. At the outset of both experiments, there were 17 rats per group. Within the first 10 days after DMBA administration, 0–2 animals from each group died, resulting in groups of 15–17 animals each. For the remainder of the experiment, there was no further loss of animals until late in the study when 4 animals died as a result of large ulcerated tumors.

Animals were examined weekly for the appearance of mammary tumors by palpation, beginning 42 days after DMBA administration. The number of animals with one or more tumors divided by the total number of animals in the group gave the tumor incidence expressed as a percentage. The average tumor number per tumor-bearing rat was also recorded. The product of the two longest perpendicular diameters of the tumor measured with calipers was used as standard estimate of the size of a lesion. This value was expressed as average tumor area (cm^2) and average tumor area (cm^2) per rat. Tumor development was followed in these rats up to 133 and 141 days after DMBA treatment, in the progesterone and R2323 experiments, respectively. Animals were sacrificed by decapitation 24 hr after the last injection of progesterone or R2323 between 08:00 and 09:00 hr and trunk blood was collected in heparinized beakers. Following separation by centrifugation, plasma was stored at −20°C until hormones were assayed.

Preparation of cytosol and membrane fractions

After decapitation of the animals, the mammary tumors were removed, freed of connective and adipose tissue and rinsed in ice-cold buffer B (10 mM Tris–HCl, pH 7.4, 1.5 mM EDTA and 10 mM thioglycerol). The tumors were then weighed and homogenized in 3 volumes (w/v) of Buffer A (25 mM Tris–HCl, pH 7.4, 1.5 EDTA, 10 mM thioglycerol and 10% glycerol) using a Polytron PT-10 homogenizer at a setting of 5 for 2 periods of 10 sec with an interval of 10 sec for cooling. The homogenates were centrifuged at 18,000 ***g*** for 15 min and the resulting supernatants were centrifuged at 105,000 ***g*** for 90 min to obtain the cytosol (supernatant) fraction. All steroid receptor binding assays were performed with fresh cytosol. The 105,000 ***g*** pellets were resuspended in 25 nM Tris–HCl, 10 mM $MgCl_2$, pH 7.4 with a Teflon–glass homogenizer and stored at −20°C until peptide binding assays.

[^{3}H] *R5020 and* [^{3}H] *estradiol-17β binding assays*

[^{3}H] R5020 (17,21-dimethyl-19-nor-pregna-4,9-diene-3-dione) and [^{3}H] estradiol-17β binding were measured using the dextran-coated charcoal assay under conditions where exchange occurs [11, 19, 20]. Duplicate 0.3 ml aliquots of adequately diluted cytosol were incubated with 0.1 ml of 4 × 10^{-8}M [^{3}H] R5020 or [^{3}H] estradiol-17β in the presence or absence of a 100-molar excess of the unlabelled steroid for 12–16 hr at 0–4°C for R5020 and 25°C for estradiol. The absence of significant degradation of estradiol receptor was evidenced by the observation of almost identical binding measured after incubation of tumor cytosol from castrated animals for 16 hr at 0° or 25°C. The degradation at 25°C did not exceed 10%. This is in agreement with results in human breast cancer cytosol fraction in which free and total E_2 receptor was measured at 0° and 25°C, respectively [20]. Similar experiments performed in uterine cytosol from various species indicate similar stability of the progesterone receptor incubated with [^{3}H] R5020 (Philibert, Ojasoo and Raynaud, unpublished data). Unbound steroid was then removed by incubation for 10 min at 0–4°C with 0.4 ml of 0.5% Norit A-0.05% Dextran-T-70 in Buffer B and centrifugation at 2000 ***g*** for 10 min at the same temperature. Four tenths of a millilitre aliquots of the supernatant were removed and after addition of 10 ml of Aquasol, the radioactivity was measured in a Beckman liquid scintillation spectrometer at a counting efficiency of 45%.

[^{125}I] *labelled ovine PRL binding assay*

Specific binding was determined in tumor membrane fraction which had been stored at −20°C for less than one week as previously

described [9, 21, 22]. Briefly, approximately 100,000 counts/min of [^{125}I] labelled ovine PRL were incubated with 300 μg of membrane fraction protein [23] in a final volume of 0.5 ml in 25 mM Tris–HCl, 10 mM $MgCl_2$ (pH 7.4) containing 0.1% bovine serum albumin. The incubation was performed in duplicate at 25°C in the presence or absence of an excess of unlabelled PRL (1 μg) for 6 hr. The incubation was stopped by addition of 3 ml of the incubation buffer. Bound and free hormones were separated by low speed centrifugation (2000 rev/min) at 4°C in an IEC PR 6000 centrifuge for 25 min. After decantation, the pellets were counted in a LKB gamma spectrometer. Specific binding was obtained by subtracting nonspecific from total bound radioactivity. This value was expressed as percentage of the total radioactivity added to the tube.

Radioimmunoassays and assay calculations

Plasma LH and PRL were measured by double-antibody radioimmunoassays [24, 25] using rat hormones (LH-I-3, LH-RP-1, PRL-I-1 and PRL-RP-1) and rabbit antisera (anti-rat LH serum I and anti-rat PRL-S-2) kindly provided by Dr. A. F. Parlow of the National Institute of Arthritis and Metabolic Diseases, Rat Pituitary Hormone Program. Radioimmunoassay data were analyzed using a program written in this laboratory and based on model II of Rodbard and Lewald [26]. Receptor assays were calculated using a program written in this laboratory. Calculations were carried out on a desk-top Hewlett-Packard 9830A calculator. All data are expressed as mean ±S.E.M. Statistical significance was calculated according to the multiple-range test of Duncan–Kramer [27].

Hormones

2,4,6,7,[^{3}H]estradiol-17β (105 Ci/mmole) was obtained from New England Nuclear while 1,2[^{3}H]R5020 and the corresponding unlabeled steroids were synthesized at the Roussel UCLAF Research Center. Ovine PRL (26 U/mg, NIH-P-S11) was generously supplied by NIAMDD. Iodination with [^{125}I] was performed using the lactoperoxidase procedure as described [28]. The specific activity of the iodinated PRL was 78 μCi/μg.

RESULTS

As illustrated in Fig. 1A, in the progesterone study, tumors first appeared in control rats 48 days after DMBA administration and the incidence of tumors gradually increased to a maximum of 87.5% at the end of the experiment (133 days). Treatment with both doses of progesterone resulted in an earlier appearance of tumors with tumors first appearing 42 days after DMBA injection. The incidence remained higher in the progesterone-treated groups during the first 90 days of the experiment to reach maximal values similar to those of controls. At day 133, the incidence was 94.1 and 78.6% for the 0.5 and 4 mg doses of progesterone, respectively. This is reflected in a reduction of the average latency period from 93 ± 3 days in the control rats to 84 ± 3 ($P<0.01$) days in the animals treated with 0.5 and 4 mg of progesterone, respectively.

The average numbers of tumors per tumor-bearing animals is shown in Fig. 1B. Control animals showed a continuous increase reaching a maximum of 4.3 ± 0.8 tumors at day 133. Although progesterone increased the number of tumors most noticeably in the mid-portion of the experiment, maximum values similar to controls were seen at day 133. In fact, 4.3 ± 0.8 and 4.7 ± 1.0 tumors, were seen for the two doses of progesterone, respectively.

In control rats, the largest average tumor size reached was 1.6 ± 0.2 cm^2 on day 91 (Fig. 1C). The value declined slightly thereafter but remained relatively stable for the remainder of the study. Progesterone at a dose of 0.5 mg caused a marked increase in the average tumor area throughout the experiment with the final value at day 133 being 2.4 ± 0.5 cm^2. The stimulatory effect of progesterone is even more evident if the tumor area/rat is examined (Fig. 1D). The lower dose of progesterone caused a very striking increase in total tumor area per rat reaching 9.6 ± 1.9 cm^2 compared to 4.5 ± 0.6 cm^2 in the control group 133 days after DMBA. The higher dose of progesterone (4 mg) had little or no effect on either tumor area or tumor area per rat [Fig. 1 (C and D)].

The inhibitory effect of R2323 on tumor development is illustrated in Fig. 2. Tumors first appeared in control rats 49 days after DMBA administration, reaching a maximum tumor incidence at day 141 of 93.8% (Fig. 2A). R2323 at doses greater than 10 μg/day caused a marked difference in the pattern of tumor development, especially after day 91, with fewer animals developing tumors than in the control group. Values at day 141 for the five increasing doses of R2323 were 70.6, 68.8, 53.3, 29.4 and 17.6%, respectively. The average latency period of 91 ± 4 days in the

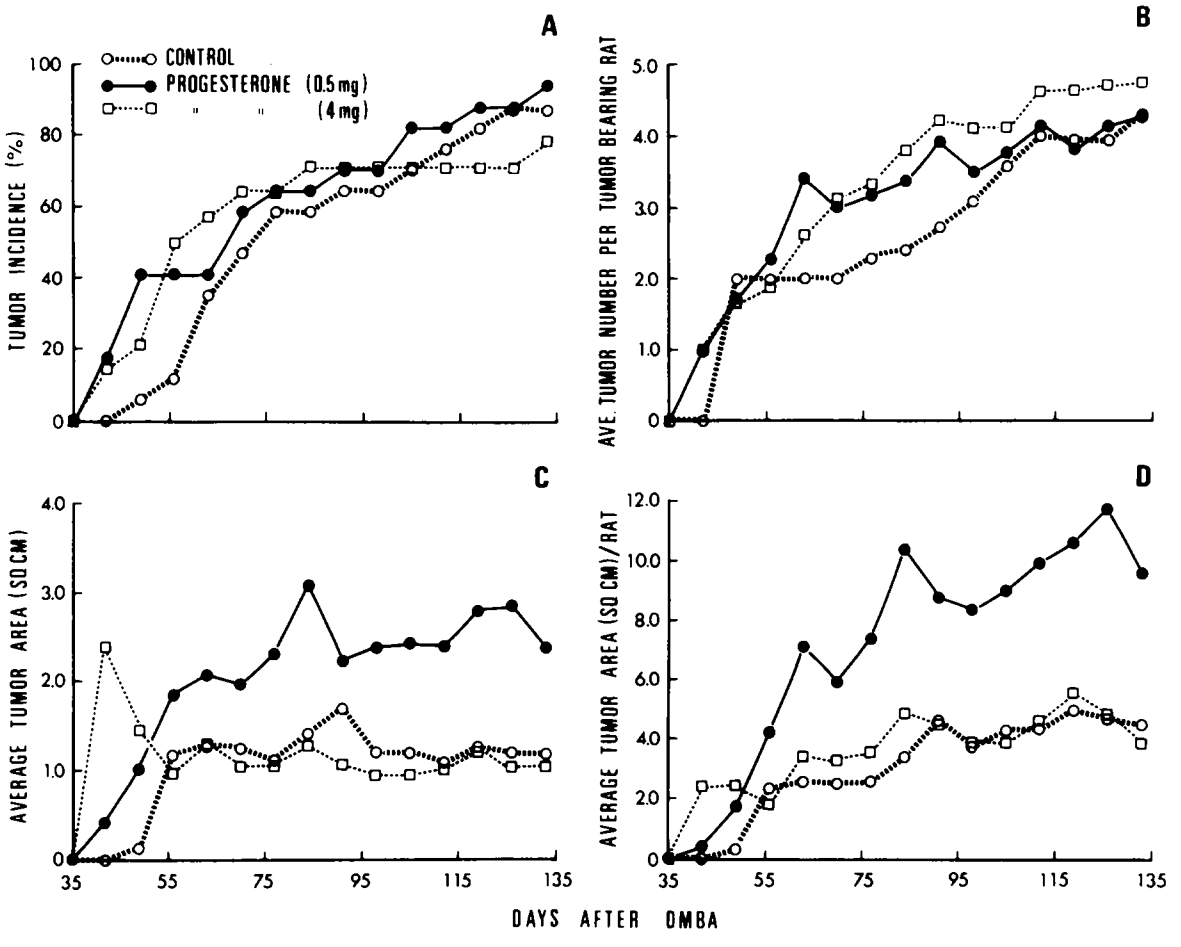

Fig. 1. Effect of daily injection of 0.5 *or* 4 mg *progesterone on the development of DMBA-induced mammary tumors. Injections began the day after DMBA was administered and continued for the next* 133 days. *Animals were examined weekly for the presence of tumors and when present, tumor area (length × width) was recorded. (A) Tumor incidence as a function of time after DMBA. (B) Average number of tumors per tumor-bearing animal. (C) Average tumor area* (cm^2). *(D) Average tumor area* (cm^2) *per rat.*

control rats was not significantly affected by treatment with R2323 with the exception of the highest dose which caused a lengthening of the latency period by 21 days ($P<0.01$).

There was a slight stimulation of tumor number (Fig. 2B) with the two lower doses of R2323, with maximum values of 4.0 ± 0.9 tumors per rat attained at day 141 in the animals injected with 2.5 μg R2323. Control rats had a maximum tumor number at day 91 of 2.9 ± 0.7 with values declining to 2.2 ± 0.5 at day 141 after DMBA. The three higher doses of R2323 progressively depressed average number with animals receiving 150 μg R2323 having only 1 tumor per rat.

The average tumor area (Fig. 2C) was markedly stimulated by the 30 μg dose of R2323 with an average tumor size of 5.0 $\pm1.8\,cm^2$ at day 98 after DMBA ($P<0.05$ compared to control). This value declined only slightly to $4.6\pm1.6\,cm^2$ at day 141. Control tumors, on the other hand, were 2.6 $\pm0.8\,cm^2$ at the same time. Doses of R2323 smaller than 30 μg were without significant effect whereas the 70 and 150 μg dose resulted in smaller tumors. For the highest dose of R2323, the tumor size increased rapidly during the last 3 weeks of the experiment. This was due entirely to 1 out of the 3 tumors in this group which accounted for over 80% of the total tumor area. Without this tumor, the tumor size would have remained low with a value of $0.8\pm0.1\,cm^2$.

The general effect of treatment on tumor size is more clearly seen in Fig. 2D where area is expressed as a function of the total number of rats per group. It can thus be seen that R2323, at the lower doses, is slightly stimulatory or has no effect whereas the two higher doses result in a greatly reduced tumor size.

The effect of treatment with progesterone

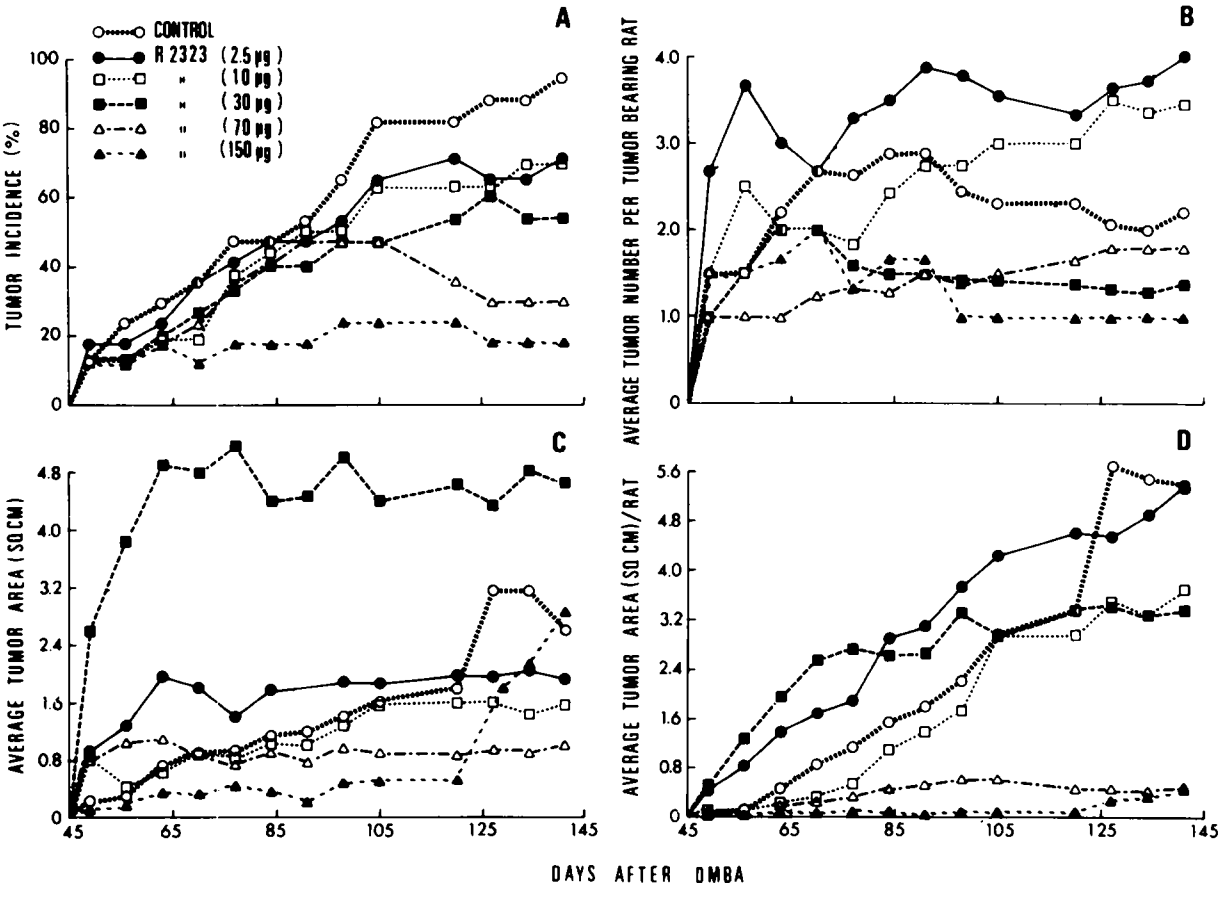

Fig. 2. Effect of treatment with increasing doses of R2323 on the development of DMBA-induced mammary tumors. Injections began the day after DMBA administration and continued for the next 141 days. *identification as in legends to Fig.* 1.

on specific binding of [^{3}H]estradiol, [^{3}H]R5020 and [^{125}I]oPRL to DMBA-induced tumors is shown in Table 1. Binding of [^{3}H]E_2 was 2.9 ± 0.3 pmole/g tissue in control tumors while progesterone, at the dose of 0.5 mg/day, increased E_2 binding to 4.6 ± 0.3 pmole/g tissue ($P < 0.01$). Binding of [^{3}H]R5020 was reduced from 12.4 ± 1.4 to 6.3 ± 1.2 pmole/g tissue by treatment with 4 mg of the steroid per day ($P < 0.01$). Binding of prolactin to the tumors was unaffected by the treatments.

Specific binding of these three hormones to tumors of rats treated with R2323 is described in Table 2. In the control group, binding of [^{3}H]estradiol and [^{3}H]R5020 and averaged 4.2 ± 0.8 and 17.6 ± 3.9 pmole/g tissue while for [^{125}I]oPRL, $4.1 \pm 0.8\%$ was bound. Although there was a tendency toward reduced values with the intermediate doses of R2323, treatment with R2323 had no significant effect on the binding of these hormones to their specific receptors.

The effects of progesterone and R2323 treatment on body weights and plasma hormone levels are summarized in tables 3 and 4, respectively. Progesterone caused a significant increase in body weight while R2323 was without effect. Because of the known prolactin dependence of DMBA tumors, the plasma levels of PRL as well as of LH were determined. Treatment with either progesterone or R2323 had no significant effect on the level of plasma PRL in animals which were killed at 0800 hr. Plasma LH, on the other hand, was reduced from 32.9 ± 3.4 ng/ml in control rats to 23.9 ± 2.8 and 17.1 ± 1.5 ng/ml by 0.5 and 4 mg progesterone, respectively (Table 3). R2323 was effective in significantly reducing plasma LH levels with doses of 10 µg/day or greater (Table 4).

DISCUSSION

These studies clearly indicate that progesterone treatment started at the time of DMBA

Table 1. *Effect of treatment with progesterone on specific binding of* [^{3}H] *estradiol-17β,* [^{3}H]*R5020 and* [^{125}I]*oPRL to DMBA-induced tumors*

Group	Number of tumors	[^{3}H]E_2 (pmole/g tissue)	[^{3}H]R5020 (pmole/g tissue)	[^{125}I] oPRL (% binding)
Control	23	2.9±0.3	12.4±1.4	5.5±1.0
Progesterone (0.5 mg)	32	4.6±0.3*	13.7±1.0	5.0±0.4
Progesterone (4 mg)	22	3.6±0.4	6.3±1.2*	5.6±0.7

*$P<0.01$ (compared to control group).

Table 2. *Effect of treatment with R2323 on the specific binding of* [^{3}H]*estradiol-17β,* [^{3}H]*R5020 and* [^{125}I]*oPRL to DMBA-induced tumors*

Group	Number of tumors	[^{3}H]E_2 (pmole/g tissue)	[^{3}H]R5020 (pmole/g tissue)	[^{125}I] oPRL (% binding)
Control	11	4.2±0.8	17.6±3.9	4.1±0.8
R2323 (2.5 μg)	14	3.0±0.3	18.1±2.3	5.0±0.6
R2323 (10 μg)	19	3.3±0.6	11.3±1.4	3.7±0.5
R2323 (30 μg)	3	4.2±1.3	14.1±5.6	2.9±1.1
R2323 (150 μg)	0	—	—	—

Table 3. *Body weights and plasma LH and PRL levels in DMBA-treated rats receiving progesterone*

Group	Number of rats	Body weight (g) Initial	Final	Difference	Plasma LH (ng/ml)	Plasma PRL
Control	16	155±2	280±6	125±6	32.9±3.4	4.7±0.9
Progesterone (0.5 mg)	15	155±2	304±8*	149±8*	23.9±2.8†	4.5±0.8
Progesterone (4 mg)	14	149±2	323±9†	174±9†	17.1±1.5†	2.9±0.4

*$P<0.05$ and †$P<0.01$ compared to control group.

Table 4. *Body weights and plasma LH and PRL levels in DMBA-treated rats receiving R2323*

Group	Number of rats	Body weight (g) Initial	Final	Difference	Plasma LH (ng/ml)	Plasma PRL
Control	15	156±3	291±7	135±8	32.8±3.6	4.0±0.9
R2323 (25 μg)	17	155±3	285±8	130±9	31.4±3.4	7.0±1.8
R2323 (10 μg)	16	151±2	284±7	133±7	28.2±3.0*	4.7±1.1
R2323 (30 μg)	15	153±3	280±6	127±6	27.9±1.9*	3.7±0.9
R2323 (70 μg)	17	159±4	292±9	133±10	23.7±2.0†	6.3±2.0
R2323 (150 μg)	17	150±2	279±6	129±6	19.9+1.4†	5.5±1.0

*$P<0.05$ and †$P<0.01$ compared to control group.

administration increases the early incidence of tumors and can have a marked stimulatory effect on tumor size. The antiprogestin R2323 on the other hand, is capable of markedly reducing the development of mammary tumors induced by DMBA when injected at daily doses of 70 or 150 μg.

Administration of progesterone has previously been reported to enhance the development of mammary tumors induced by DMBA [7, 8, 29]. On the other hand, Terenius [30] reported that daily treatment for 13 days with approximately 4 mg progesterone started at the time DMBA was administered had no effect on the number or size of tumors. It has also been reported that when progesterone is given early enough, it can have an inhibitory effect on the development of mammary tumors [10, 31]. Injection of 4 mg of progesterone 20 days before and 20 days after DMBA was capable of significantly reducing the percentage of rats with tumors as well as the number and size of tumors [31].

The present experiments show that progesterone can stimulate tumor development when treatment with the steroid is started the day after DMBA administration. Although it is possible that the small increase of the level of the estrogen receptor observed after treatment with the 0.5 mg dose of progesterone might be partly involved in the increased tumor incidence and growth, the concomitant inhibitory effect of this dose of progesterone on LH secretion with probable decreased ovarian function make it likely that progesterone exerts its stimulatory action by a direct interaction with its receptor in the tumor tissue [11]. It seems unlikely that progesterone is acting via an effect on prolactin secretion or on the level of prolactin receptor since both parameters remained unchanged after progesterone treatment.

The compound R2323 has very weak estrogenic activity in the rat and mouse (vaginal cornification = 1–2/1000 the potency of estradiol-17β; uterotropic activity of 1/10,000 that of ethinyl estradiol) and moderate progestomimetic and weak androgenic activities. In terms of anti-progestational activity, treatments with 2–5 mg/day, was found to completely inhibit the endometrial response induced by 200μg of progesterone and 0.2 mg of R2323 prevented the maintenance of pregnancy which would normally be observed following treatment with 15 mg of progesterone [16]. In addition, R2323 can inhibit the basal level of LH as well as the LH response to LHRH with as little as 10 μg per day (Ferland and Labrie, unpublished observations).

When injected daily for 141 days following DMBA administration, R2323 at doses of 70 and 150 μg was capable of markedly reducing tumor incidence and size. This effect is best illustrated in Fig. 2D. It should be mentioned that there were no significant effects of any dose of R2323 on receptor levels for 17β-estradiol, R5020 or PRL in tumor tissue. R2323 also had no significant effect on plasma prolactin levels in rats killed at 08.00 hr on day 141 after DMBA administration indicating the antiprogestin is not acting via a decrease in circulating prolactin levels.

Treatment with the higher dose of progesterone reduced the number of progestin binding sites in DMBA-induced tumors, as has previously shown in the uterus [17]. This effect of progesterone is likely to be due to translocation of the cytosol receptor into the nucleus. However, treatment with the antiprogestin R2323 was without significant effect on this parameter (Table 2).

It is well known that DMBA-induced mammary tumors are dependent upon prolactin and estrogens for growth [1–3, 5, 6, 32]. Recently, we have reported the potent antitumor activity of a new antiestrogen, RU16117 (11α-methoxyethinyl estradiol). At a daily dose of 8 μg, this steroid was capable of completely preventing the development of mammary tumors [4, 22]. We have also shown that RU16117 was capable of inducing the regression of established mammary tumors in a fashion parallel to the effect of ovariectomy [9]. Since RU16117 caused a reduction in the concentration of specific hormone receptors to bind estradiol in the tumor, it is possible that the anti-tumor effect of RU16117 may be at least partly due to an effect at the level of the tumor tissue itself. R2323, however, has no inhibitory effect on the levels of receptor for estradiol, progesterone or prolactin in the tumor tissue. Since R2323 is a potent inhibitor of gonadotropin secretion (Ferland and Labrie, unpublished observations), it seems that one likely site of the inhibitory action of the anti-progestin is at the hypothalamic pituitary level. In addition, R2323 binds to the progesterone receptor with an affinity similar to that of progesterone. It is thus possible that part of its action is exerted by competition with the action of progesterone on the tumor in agreement with data obtained at the uterine level [16].

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