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CHARACTERIZATION OF ESTROGEN-INDUCED PROGESTIN BINDING IN CYTOSOL OF THE R3327 PROSTATIC CARCINOMA OF THE RAT

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Summary—High affinity binding of the synthetic steroids methyltrienolone (R1881) and promegestone (R5020) to cytosol protein from the Dunning (R3327) experimental prostatic carcinoma of the rat was investigated. Animals bearing tumours of approx 1.5 cm mean diameter were either left untreated, or were administered diethylstilbestrol diphosphate (DESP) in the drinking water in doses close to those used clinically for the treatment of human prostatic carcinoma. Tumours were excised after 10–40 days, and binding of [^{3}H]R1881 and [^{3}H]R5020 to tumour cytosol was characterized using Scatchard analysis, sucrose density gradient centrifugation, and steroid competition, under conditions optimal for the conservation and assay of progesterone receptor. Both ligands were bound in much higher concentrations by cytosol from DESP-treated tumours than from untreated tumours. Binding was of high affinity ($K_d \simeq 1$ nM), was specific for progestins, and sedimented in peaks at ~8S and ~4S in sucrose density gradients. We conclude the DESP treatment of rats bearing the R3327 prostatic carcinoma induces synthesis of progesterone receptor in this tumour.

INTRODUCTION

The R3327 prostatic adenocarcinoma, which arose spontaneously in the dorsolateral prostate of an aged Copenhagen rat in 1961 [1], has many of the characteristics of human prostatic carcinoma, and has been widely used as a model for the human disease. It has a similar enzyme profile, contains androgen receptor (AR) and estrogen receptor (ER), and growth can be retarded by castration and estrogen treatment [2]. However, as a fraction of the cell population is not androgen-sensitive, the tumour eventually relapses from hormonal control and growth is resumed [2]. In an investigation of the effects of hormonal manipulation on the concentration and cellular distribution of binding sites for methyltrienolone (R1881) in this tumour model, we observed elevation of the concentration of cytosol sites binding this synthetic steroid after treatment of the host animals with diethylstilbestrol diphosphate (DESP) [3]. Although the assay conditions in these experiments were unfavourable for the conservation of progesterone receptor (PgR), the steroid specificity demonstrated in competition studies suggested that at least part of the elevated binding observed was due to progestin-binding protein(s). We have now characterized more completely the binding of both R1881 and the synthetic progestin promegestone (R5020) to cytosol protein from untreated and DESP-treated R3327 tumours under conditions which conserve PgR. We conclude from this characterization that treatment of animals bearing this tumour with DESP is able to induce progestin-binding protein with the properties of PgR.

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Abbreviations: AR, androgen receptor; BSA, bovine serum albumin; DCC, Dextran-coated charcoal; DES, diethylstilbestrol, DESP, diethylstilbestrol diphosphate (sodium salt); DHT, 5α-dihydrotestosterone; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ER, estrogen receptor; PgR, progesterone receptor; PMSF, phenylmethylsulfonylfluoride; RBA, relative binding affinity; R1881, methyltrienolone; R5020, promegestone; TA, triamcinolone acetonide.

EXPERIMENTAL

Animals and tumours

Copenhagen × Fischer F1 hybrid male rats were obtained from Dr N. Altman, The Papanicolaou Cancer Research Institute at Miami, Inc., by the courtesy of the National Prostatic Cancer Project (NIH). Animals had received subcutaneous tumour implants by trocar on the flanks at the PCRI, or were implanted in our own laboratory. When tumours became palpable, growth was monitored by weekly caliper measurement of the longest diameter and the diameter at right-angles to it. Treatment was begun when the mean diameter reached approx 1.5 cm, and tumour size was monitored until the animals were killed.

Experimental animals were treated with the sodium salt of diethylstilbestrol (α, α′-diethylstilbenediol) diphosphate (DESP) in the drinking water at a concentration of 1.6 μg/ml [4]. Control animals were left untreated. Intake of DESP was monitored by the use of calibrated water bottles. For the animals used in these experiments, the length of treatment ranged from 10–40 days, and the mean daily intake of DES

ranged from 7–17 μg per animal. This is equivalent to approx 2.5 mg/d in a 70 kg man, and is close to the dose used clinically for the treatment of prostatic carcinoma. In all, five tumours from treated animals, and four from untreated control animals were used.

After the treatment period, each tumour was excised, the capsule was removed, and a representative slice was fixed for histological examination. The remainder was rinsed in homogenization buffer, cut into pieces and snap frozen in foil containers in liquid nitrogen, where they were stored until assay (not longer than 20 weeks).

Rat uteri were used in order to compare the affinity of the binding of progestins in uterine cytosol with that in R3327 tumour cytosol. Uteri were excised from mature rats weighing approx 250 g which had been ovariectomized 3 days previously to eliminate endogenous ovarian steroids. All subsequent procedures were identical to those used for the tumours.

Isotopes and chemicals

[Methyl-^{14}C]methylated ovalbumin and γ-globulin, [^{3}H]methyltrienolone (R1881; Sp. act. 87.0 Ci/mmol), [^{3}H]promegestone (R5020; Sp. act. 87.0 Ci/mmol), and corresponding radioinert steroids were obtained from New England Nuclear Corp. On arrival the labelled steroids were diluted in redistilled benzene–ethanol (9:1, v/v) and stored at 0°C for not more than 3 months. Other unlabelled steroids, phenymethylsulfonylfluoride (PMSF), dithiothreitol (DTT) and BSA were obtained from Sigma Chemical Co. (St Louis, MO); Dextran T70 from Pharmacia (Montreal); charcoal (Norit-A) from Matheson, Coleman and Bell. The scintillators used were either PCS(Amersham) or PCS–toluene (2:1, v/v).

Buffers and solutions

The pH of all buffers was 7.4. Tris: 10 mM Tris–HCl; TE: 10 mM Tris, 1.5 mM EDTA; Buffer A: 10 mM Tris, 1.5 mM EDTA, 1 mM DTT, 0.1 mM PMSF; Buffer B: buffer A + 10 mM $Na_2MoO_4(2H_2O)$ + 40% glycerol; Buffer C: buffer A + 10 mM $Na_2MoO_4(2H_2O)$ + 10% glycerol; DCC suspension: 0.33% charcoal, 0.033% Dextran in buffer A + 10 mM $Na_2MoO_4(2H_2O)$ + 20% glycerol.

Homogenization and cytosol preparation

All tissue handling and assay procedures were carried out at 0–4°C with pre-cooled equipment, glassware and buffer solutions. Frozen tissue was pulverized in a Thermovac pulverizer (Thermovac Industries Corp. Copiague, N.Y.) cooled with liquid nitrogen and then homogenized in buffer A (100 mg/ml) using a Polytron P-10 homogenizer (Brinkman Instruments Inc.) for 2 bursts of 20 s (setting 3) with a 30 s cooling interval. The homogenate was centrifuged at 3000 *g* for 10 min to yield a crude supernatant (SN), which was immediately adjusted to contain 10 mM molybdate by the addition of 1/10 volume of 110 mM sodium molybdate in buffer A. The crude SN was then centrifuged at 145,000 *g* for 1 h to separate the cytosol (3–5 mg protein/ml). An aliquot of cytosol was taken for protein assay [5].

Scatchard analysis

In order to compare the binding characteristics of R1881 and R5020 to progestin-binding protein(s), Scatchard analysis was carried out on cytosol of the same tumours and of the same uterine preparation using both ligands. 8% Glycerol and 10 mM sodium molybdate were present during all incubations so as to conserve progesterone receptor (PgR) [6]. Excess radioinert cortisol and 5α-dihydrotestosterone (DHT) were added to all incubations using R5020 as ligand, in order to prevent binding to glucocorticoid and androgen receptor [7, 8]. Replicate 200 μl aliquots of cytosol were incubated with 50 μl buffer B containing [^{3}H]R5020, cortisol and DHT to give final concentrations of 1–10 nM [^{3}H]R5020 with 100-fold concentration of cortisol and 10-fold concentrations of DHT. Parallel sets of aliquots were incubated with 100-fold radioinert R5020 added to permit correction for low-affinity binding.

The method of Ekman was used to investigate R1881 binding [9]. This uses excess triamcinolone acetonide (TA) to compete out high affinity binding of [^{3}H]R1881 to PgR, as described by Zava [10]. Parallel sets of 200 μl aliquots of cytosol were incubated with 50 μl buffer B with 1–10 nM [^{3}H]R1881 and with the same concentrations of [^{3}H]R1881 with 500-fold excess TA. The difference in binding values between the two sets of aliquots represents high-affinity binding to PgR.

All incubations were carried out for 2h at 0–4°C, and were terminated by the addition of 500 μl DCC suspension. After vortexing, separation of free from bound steroid was carried out by incubation for 15 min followed by centrifugation at 2500 *g* for 10 min. The supernatant was then recentrifuged. An aliquot (400 μl) of each final supernatant was counted in 10 ml PCS scintillator for 20 min or to 2% error.

All Scatchard analyses were carried out with triplicate aliquots under each condition at each of 5 ligand concentrations. The B_{max} and K_d were calculated using the programme of Schwarz [11] on a Hewlett–Packard HP-97 calculator.

Steroid competition studies

These were carried out on cytosol from DESP-treated tumours using the same conditions as for the Scatchard analysis. Labelled ligand concentrations were 10 nM for [^{3}H]R1881 and 5 nM for [^{3}H]R5020 in the presence of excess cortisol and DHT. Parallel sets of duplicate aliquots were incubated with added radioinert steroid competitors at 1, 5, 25, 50 and 100 times that of the labelled ligand. The competitors used to compete with both ligands were R5020, R1881, progesterone, norethindrone, cyproterone ac-

etate and estradiol. Testosterone, DHT and cortisol were also used to compete with [^{3}H]R1881. High affinity binding of [^{3}H]R5020 and [^{3}H]R1881 was defined as that binding which was competed out by 100-fold radioinert R5020 and R1881 respectively. The percent reduction in high affinity binding brought about by each concentration of each competitor was calculated. Relative binding affinity (RBA) was defined as the ratio of [ligand]:[competitor] necessary to reduce high affinity binding by 50%.

Sucrose density gradient centrifugation

The method used was based on that of Bevins and Bashirelahi [6]. Cytosol was prepared as for Scatchard analysis, and was incubated with 10 nM [^{3}H]R5020 in the presence of 1 μM cortisol and 50 nM DHT $\pm$ 1μM R5020 for 2 h at 0°C. Free steroid was removed with a DCC pellet (15 min) and 0.3 ml aliquots of the separated supernatant, containing 0.3–1 mg protein, were applied to duplicate gradients of 5–20% sucrose in buffer C. ^{14}C-Labelled γ-globulin and ovalbumin were used as internal markers (7S and 3.6S, respectively) Gradients were spun at 220,000 g_{av} for 3 h in a vertical (VTi65) rotor, and 15 drop fractions were collected into counting vials and counted in 0.25 ml distilled water/10 ml PCS–toluene scintillator. Recovery was 70–95% of radioactivity applied.

RESULTS

Scatchard analysis

Significant concentrations (> 150 fm/mg protein) of high affinity binding of both ligands were observed in the cytosol from treated tumours. The B_{max} values were approx 20% of those observed in the rat uterus using the same ligands under the same conditions. In both the uterus and the treated tumours, the B_{max} values were 8–11% higher when [^{3}H]R1881 was used as ligand than when [^{3}H]R5020 was used, but binding affinity was slightly lower with [^{3}H]R1881 (Figs 1 and 2). No high affinity binding of [^{3}H]R5020 was detected in untreated tumours, but with [^{3}H]R1881, high affinity binding was observed in the same tumours at concentrations between 20 and 30 fm/mg cytosol protein, with K_d's 0.9–2.0 nM.

Sucrose density gradient centrifugation

SDGC analysis of cytosol from an untreated tumour after incubation with [^{3}H]R5020 under conditions which conserve PgR and eliminate binding to AR and glucocorticoid receptor showed a small peak of saturable binding at ~8S. In a parallel analysis on a DESP-treated tumour, this peak was more than 10-fold larger, and in addition a smaller peak at ~4S was observed (Fig. 3).

Competition studies

When [^{3}H]R5020 was used as ligand in the competition studies, radioinert R5020, R1881, progesterone and norethindrone gave parallel competition curves and had RBA's ranked in that order (Fig. 4). All four reduced high-affinity binding of [^{3}H]R5020 by > 50% when present at five times the concentration of this ligand. Cyproterone acetate and estradiol had much lower RBA's, reducing high affinity binding of [^{3}H]R5020 by 50% when present at 10-fold the concentration of ligand. Very similar results were obtained using the [^{3}H]R1881 $\pm$ TA method. The same four steroids gave similar competition curves and had similar RBA's ranked in the same order as that obtained with [^{3}H]R5020. Cyproterone acetate, estradiol and the two androgens used as competitors had much lower RBA's (Fig. 5). Cortisol did not compete at all for high affinity binding of [^{3}H]R1881.

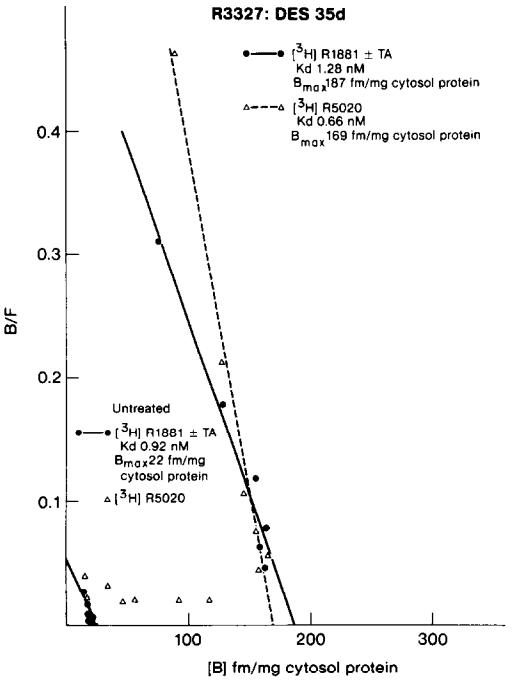

Fig. 1. Scatchard plots of high affinity binding of [^{3}H]R5020 and [^{3}H]R1881 $\pm$ TA in treated and untreated R3327 tumours. Cytosol from the same tumours was incubated with both ligands at concentrations ranging from 1–10 nM for 2 h at 0–4°C. [^{3}H]R5020 was used in the presence of 100-fold concentrations of cortisol and 10-fold concentrations of DHT. Parallel sets of aliquots were incubated with 100-fold concentrations of R5020 in addition to cortisol and DHT to permit correction for low-affinity binding. Parallel sets of aliquots were incubated with [^{3}H]R1881 either alone or in the presence of 500-fold excess TA to compete out PgR. High affinity binding to PgR was obtained by subtracting binding in the presence of TA from total [^{3}H]R1881 binding.

DISCUSSION

The data provide strong evidence that the elevated binding of [^{3}H]R1881 in R3327 tumours from estrogen-treated animals which we have previously reported [3] is indeed due to progesterone receptor. Using two ligands under conditions optimal for PgR quantitation, high affinity binding specific for

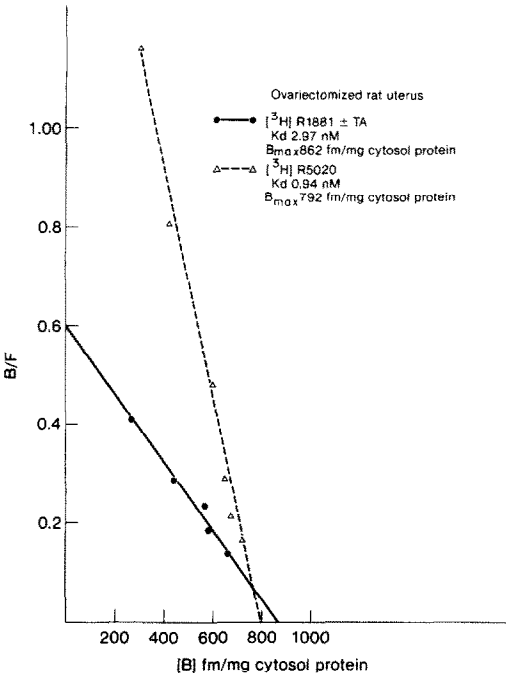

Fig. 2. Scatchard plots of high affinity binding of [^{3}H]R5020 and [^{3}H]R1881 ± TA in uterus from a mature rat ovariectomized 3 days previously. Aliquots of the same cytosol were incubated using both [^{3}H]R5020 and [^{3}H]R1881 as ligands under the same conditions as in Fig. 1.

progestins was observed. The affinity for both ligands was similar to that observed for PgR in the rat uterus assayed under the same conditions. After incubation of treated tumour cytosol with [^{3}H]R5020 under

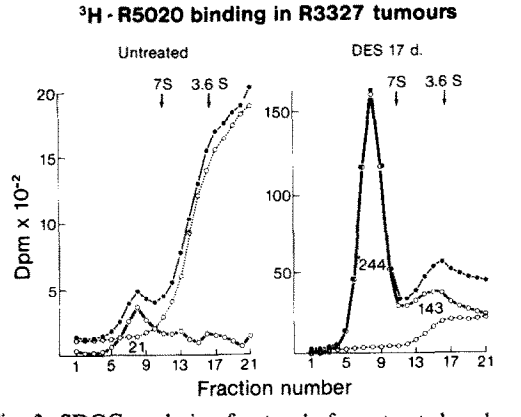

Fig. 3. SDGC analysis of cytosols from treated and untreated R3327 tumours after incubation with 10nM 3H[R5020] in the presence of 1 μM cortisol and 50nM DHT for 2h at 0°C. Parallel incubations were carried out in the presence of 1 μM radioinert R5020 to compete out saturable binding. Aliquots of cytosol were applied to 5–20% sucrose gradients and spun at 220,000g_{av} for 3h in a VTi65 rotor. ^{14}C-Labelled γ-globulin and ovalbumin were used as internal markers. Total [^{3}H]R5020 ●——●, non-saturable activity O---O, saturable binding O——O. Each line is the mean from two separate gradients on the same cytosol preparation. Figures under the peaks of saturable binding represent total high-affinity binding per mg protein applied. Note the different scales on the ordinates. Reprinted from ref. [3], with permission from Raven Press, New York.

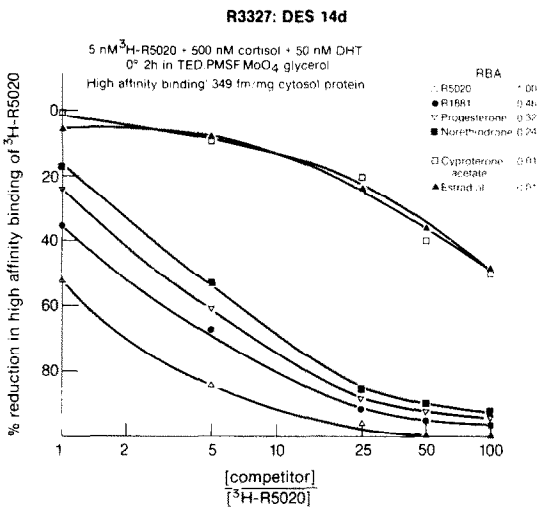

Fig. 4. Steroid competition for high-affinity binding of [^{3}H]R5020 in treated R3327 tumour cytosol. Cytosol was incubated with 5 nM [^{3}H]R5020 in the presence of 500 nM cortisol and 50 nM DHT, alone and with competitors added at concentrations ranging from 5–500 nM, for 2 h at 0–4°C. Relative binding affinity (RBA) is defined as the ratio of the concentration of ligand to that of competitor required to reduce high-affinity binding by 50%.

conditions which eliminate binding to androgen and glucocorticoid receptor, saturable binding sedimented in peaks at approx 8S and 4S on sucrose density gradient centrifugation in much larger amounts than in untreated tumour cytosol. Progesterone binding in cytosol of some R3327 tumours after treatment with estradiol benzoate treatment was also reported by Ip *et al.* [12], but the binding was not completely charac-

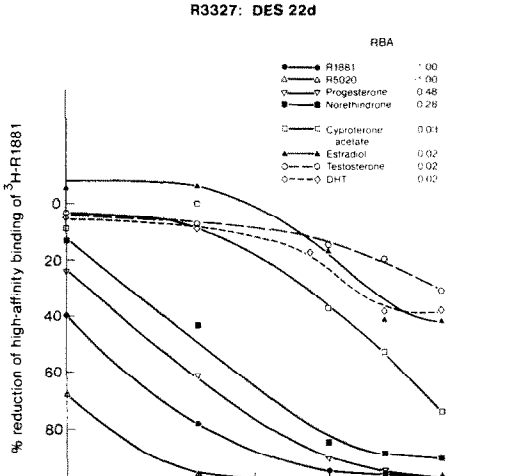

Fig. 5. Steroid competition for high-affinity binding of [^{3}H]R1881 ± TA in treated R3327 tumour cytosol. Cytosol was incubated with 10 nM [^{3}H]R1881 in the presence and absence of 5 μM TA, alone and with competitors added at concentrations ranging from 10 nM–1 μM for 2 h at 0–4°C. High-affinity binding values in the presence of TA were subtracted from high-affinity binding values in the absence of TA to obtain binding values for PgR. RBA is defined as in Fig. 4.

terized. A number of other investigators have looked for PgR in R3327 tumours. Using [^{3}H]R5020 as ligand, Markland *et al.*. [13], Dahlberg *et al.* [14], and Heston *et al.* [15] were unable to detect PgR in untreated tumours: however, the latter group of authors observed high affinity binding of this ligand in tumours which had resumed growth after castration. Thompson and Chung found that high affinity binding of [^{3}H]R1881 in the cytosol of a hormone independent R3327 tumour line was suppressed by TA, suggesting that some PgR was present [16]. We were able to detect a small amount (<30 fm/mg cytosol protein) in untreated tumour cytosol by Scatchard analysis using the [^{3}H]R1881 $\pm$ TA method, but not in the same cytosols using [^{3}H]R5020 as ligand. On the other hand, the latter ligand gave a small suppressible 8S peak after sucrose density gradient centrifugation of incubated cytosol from another untreated tumour. It seems likely that small amounts of PgR are present in at least some untreated R3327 tumours, and that detection may depend on minor differences in methodology.

The data also provide further validation for the use of the [^{3}H]R1881 $\pm$ TA method for the quantitation of PgR. The steroid specificity of the high affinity binding was identical with that of [^{3}H]R5020 high affinity binding, with very similar RBA's for the competing steroids for both ligands. B_{max} values were very similar for both ligands in both the R3327 tumours and in the rat uterus. The possibility that the slightly higher B_{max} and slightly lower affinity obtained when the [^{3}H]R1881 $\pm$ TA method was used is due to interference by glucocorticoid and/or mineralocorticoid receptor must be considered. R1881 has an affinity for glucocorticoid receptor approximately seven-fold less than that for PgR, when assayed under similar conditions to those we have used [17]. If present in significant concentrations, glucocorticoid receptor could therefore interfere with this method of assay. However glucocorticoid receptor has not been demonstrated in the R3327 tumour [4], and in our investigation cortisol did not compete with [^{3}H]R1881 binding in tumour cytosol. Moreover, similar slight differences in B_{max} and K_d for the binding of the two ligands were observed in the mature rat uterus, which has not been shown to contain cytosolic glucocorticoid receptor [18]. The affinity of [^{3}H]R1881 for mineralocorticoid receptor is somewhat less than that for glucocorticoid receptor [17]. Markland and Lee [13] found that dexamethasone, which has an equivalent affinity to that of TA for glucocorticoid receptor, and a higher affinity than TA for mineralocorticoid receptor [17], showed virtually no ability to compete for [^{3}H]R1881 binding in the R3327 tumour. It is therefore unlikely that either glucocorticoid or mineralocorticoid receptor contributed to [^{3}H]R1881 binding in our investigation.

This investigation adds to the growing amount of evidence that prostatic tissue in several species contains PgR. Moreover, it extends the observation of Frenette *et al.* on the dog prostate that estrogen exerts an inductive effect on PgR synthesis [19], and supports the concept that the prostate should be regarded as a potential target for direct estrogenic and progestogenic action.

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