J. Steroid Biochem. Molec. Biol. Vol. 37, No. 5, pp. 631–641, 1990
Printed in Great Britain.

0960-0760/90 $3.00 + 0.00

ESTROGEN RECEPTORS, PROGESTIN RECEPTORS AND DNA SYNTHESIS IN THE MACAQUE ENDOMETRIUM DURING THE LUTEAL–FOLLICULAR TRANSITION*

MARYANNE C. MCCLELLAN,† SUSANNAH RANKIN, NEAL B. WEST and ROBERT M. BRENNER
Reed College (M.C.M.), Portland OR 97202, Tufts University (S.R.), Boston, MA 02109 and Oregon Regional Primate Research Center (N.B.W., R.M.B.), Beaverton, OR 97006, U.S.A.

(Received 11 June 1990)

Summary—We have suggested that in the nonhuman primate endometrium, stromal cells might play a role in mediating the effects of estrogen on the epithelium, especially during the luteal–follicular transition (LFT) when target cells normally escape from the inhibitory influence of progesterone (P). We now report that like estrogen receptors (ER), endometrial progestin receptors (PR) are detectable only in stromal cells until the fifth day of the LFT. With a technique that combined immunocytochemistry and autoradiography on the same sections, we characterized the cellular distribution of ER or PR coincidentally with the localization of [^{3}H]thymidine taken up *in vitro* by endometria from monkeys undergoing an LFT. DNA synthesis in the glands of the upper endometrium was E_2-dependent, but the distribution of [^{3}H]thymidine was not positively correlated with the presence of ER or PR. Readministration of P to animals on days 3 or 4 of the LFT significantly reduced the [^{3}H]thymidine labeling index of the glandular epithelium and caused stromal ER to decline, but P did not block the eventual appearance of ER in epithelial cells on day 5 of the LFT. Thus, E_2 stimulated DNA synthesis in epithelial cells that lacked ER, and P suppressed DNA synthesis in these cells even though PR was only detected in the stroma when P treatment began. These data are consistent with a role for endometrial stromal cells in mediating the effects of E_2 and P on the epithelium during the LFT.

INTRODUCTION

The primate menstrual cycle is characterized by dramatic shifts in serum concentrations of estradiol (E_2) and progesterone (P) which provoke equally remarkable cellular changes in the endometrium. An important shift in the balance of influence of E_2 and P on the endometrium occurs when serum P levels fall rapidly at the end of the luteal phase of a nonfertile cycle while concentrations of E_2 remain relatively constant [1]. In the natural menstrual cycle, the uppermost regions of the endometrium are sloughed in the menstrual flow, and the surface of the remaining tissue heals. In response to E_2, the glandular elements of the surviving endometrial tissue proliferate to regenerate the complete endometrium [2]. Bartelmez [3] described four distinct zones in the endometrium of the rhesus macaque to account for the consistent morphological gradient he observed from the lumenal surface (zone I), to the region adjacent to the myometrium (zone IV). He reported that mitotic activity was confined to zones I–III before ovulation, and that zone IV was mitotically inactive until shortly after ovulation. Recently, Padykula [4] has confirmed these observations with [^{3}H]thymidine autoradiography.

Estrogen receptors (ER) are presumed to play a role in the endometrial proliferative response to E_2 because concentrations of ER parallel E_2 action, and substances such as antiestrogens and P which interfere specifically with ER action or lower ER concentrations also depress estrogen-dependent growth [5–7]. In the adult macaque endometrium, the antagonistic effects of E_2 and P on cell proliferation and the synthesis of ER and PR as well as the separated pathways of subcellular differentiation induced by these hormones are well characterized [8–10]. In a previous study, we used E_2 and P capsules to create an artificial luteal–follicular transition (LFT) in spayed monkeys and reported that endometrial ER levels, which were low at the end of P

*A part of this work was presented at the *69th Annual Meeting of The Endocrine Society*, Abstr. No. 195.

†To whom correspondence should be addressed: Dr M. McClellan, Department of Biology, Reed College, 3203 S.E. Woodstock Blvd., Portland, OR 97202, U.S.A.

treatment, increased steadily during the 7 day period following removal of the P-capsule, though serum E_2 levels were constant. Because the various endometrial zones are not fully re-established during the LFT, we studied the distribution of ER in the upper glands (zone II/III). Immunocytochemically detectable ER was exclusively located in endometrial stromal cells until day 5, and the appearance of epithelial ER on day 5 coincided with an 8-fold increase in the epithelial mitotic index [11]. This suggested that progression through G1 of the cell cycle and commitment to DNA synthesis occurred in epithelial cells that lacked ER.

In the current study, we used a combined immunocytochemical/autoradiographic (ICC/AR) method to localize ER or PR together with [^{3}H]thymidine in endometria from monkeys experiencing an LFT. We also mapped the general distribution of endometrial PR during days 0–5 of the LFT. We found that ER and PR were present almost exclusively in the stroma when a significant estrogen-dependent increase in [^{3}H]thymidine uptake occurred in the glandular epithelium. Moreover, if P was readministered to animals on days 3 or 4 of the LFT when PR was only detected in the stroma, epithelial DNA synthesis measured on day 5 was inhibited, and stromal but not epithelial ER was suppressed. The results support the hypothesis that stromal cells might mediate effects of both E_2 and P on the epithelium during the LFT.

EXPERIMENTAL

Animals

Adult female cynomolgus macaques (*Macaca fascicularis*) were housed and cared for under the supervision of the veterinary staff of the Oregon Regional Primate Center according to the guidelines established by the National Institutes of Health and the Animal Welfare Act. Twenty animals were ovariectomized under Fluothane/nitrous oxide anesthesia and allowed to rest 2–3 months before the experiment. Silastic™ tubing (Dow Corning: i.d., 0.34 cm; o.d., 0.46 cm) was filled with crystalline steroid (Steraloids, Wilton, N.H.), and the ends were sealed with the Silastic silicone type A medical adhesive. Each animal was implanted with a 2 cm E_2-filled Silastic™ capsule while chemically restrained with ketamine. Fourteen days later a 6 cm P-filled implant was inserted into each animal and allowed to remain in place for 14 days to produce an artificial menstrual cycle. In 17 of these animals, an artificial LFT was induced by removing only the P capsule (designated as day 0). Menses occurred 2–3 days following removal of the P implants. The uterus was removed from 9 animals on either day 4 ($n = 1$), 4.5 ($n = 3$) or 5 ($n = 5$) following removal of the P implant (E_2-maintained animals). In 8 animals, the P implant was reinserted on day 3 or 4 of the LFT (P-treated animals), and uteri were collected either 1 ($n = 3$), 2 ($n = 2$) or 3 ($n = 3$) days later. Control tissue was obtained from 3 animals 5 days after removal of both E_2 and P implants (E_2 and P withdrawn animals). Concentrations of E_2 and P were determined in serum obtained on the day of surgery. Steroid radioimmunoassay procedures have been previously described [12,13] except that different antisera for E_2 [14] and P [15] were used.

Tissue

Uteri were separated from the cervix and quartered along the longitudinal axis. The endometrium from two of the quarters was cut into thin (less than 1 mm) cross-sectional slices with a razor blade. Two slices were fixed, three were frozen immediately and the rest were incubated in 1 μCi/ml [^{3}H]thymidine in Trowell's medium (pH 7, 25 mM HEPES) for 60 min, at 37°C in an atmosphere of 60% oxygen, 33% nitrogen and 7% carbon dioxide. After incubation, the slices of tissue were placed in a drop of OCT™ (Tissue Tek, Miles, Elkhart, Ind.) and frozen in liquid propane for immunocytochemistry of ER and PR. The endometrium from the remaining two quarters was removed with iris scissors and analyzed for ER quantities as described below. Details of procedures for fixation and morphological evaluation, biochemical measurement of ER and quantitation of mitotic indices (MI) were identical to those described previously [8, 11, 16]. A complete set of frozen blocks was available from the previously published study of ER during the LFT [11]. These, as well as tissues collected for the current experiments, were used to establish the general pattern of PR distribution during the LFT (days 0–5).

ER binding and exchange assay

Tissue was homogenized in 10 vol (w/v) TE buffer [10 mM Tris–(hydroxymethyl) aminomethane HCl, pH 7.4, 1.5 mM ethylenedinitrilotetraacetic acid] containing 1 mM dithiothreitol

in an ice bath by means of Duall tissue grinders, size 22 (Kontes Glass Company, Vineland, N.J.). All subsequent procedures were done at 0–4°C. The homogenate was centrifuged at 1000 *g* for 10 min to separate the cytosol from the crude nuclear fraction. Three hundred microliter aliquots of the cytosols were mixed with [^{3}H]Moxestrol [R2858; 11 β-methoxy-17-ethynyl-1,3,5,(10)-estratriene-3,17-diol; 79-87 Ci/mmol; New England Nuclear (Boston, Mass.); 23 nM final concentration] in the absence (A) or presence (B) of 100-fold radioinert Moxestrol and were incubated for 3 h at 0°C. The cytosols were then added to charcoal pellets obtained by centrifugation from 1 ml of a dextran charcoal suspension (1.25% charcoal and 0.625% dextran in TE buffer). The cytosols were mixed gently with the charcoal for 10 min and then centrifuged at 8000 *g* for 10 min. The supernatants were then drawn off from the charcoal pellets, mixed with 5 ml of Atomlite™ (New England Nuclear Corp.), and the radioactivity was determined in a scintillation counter. The crude nuclear pellets, prepared as described above, were washed 3 times with 2 ml of TE buffer each time and by centrifugation at 1000 *g* for 10 min. Aliquots (300 μl) of washed nuclear resuspensions were mixed with [^{3}H]Moxestrol as above. After incubation for 1 h at 37°C, nuclei were washed 4 times: first with 1 ml of 0.5% Triton X-100 in TE buffer, then 3 times with 1 ml of TE. The washed nuclear pellets were dissolved in 1 ml Soluene 100™ (Packard Instrument Company, Downers Grove, Ill.) at 70°C for 1 h. Five milliliters of Atomlite solution were added, and the radioactivity was determined as above. The specific receptor, defined as the difference between total (A) and nonspecific (B) binding, is expressed as femtomoles per milligram of DNA.

Immunocytochemistry and autoradiography

Immunocytochemistry of ER was done as described previously [11, 17]. Immunocytochemistry for PR was accomplished with the same protocol as for ER except that the antiprogesterone receptor antibody, B-39 (generously provided by Dr Geoffrey Greene) was diluted to a concentration of 0.5–0.1 μg/ml. That this antiPR recognizes the macaque PR has been previously reported [10, 18]. This concentration range was 10–50 times the lowest concentration necessary to detect PR in serial sections of endometria from estrogenized animals shown to have high levels of both stromal and epithelial PR. Approximately 70–80, 6 μ, serial sections were adhered to gel coated slides (two sections per slide). Fewer slides were prepared from fresh frozen blocks not incubated in [^{3}H]thymidine. Alternating slides were fixed and immunostained for ER or PR. Several slides from each block were either exposed to antiantigen B of Timothy Grass Pollen (AT) or buffer in place of the antireceptor antibody. For [^{3}H]thymidine labeled blocks, alternating pairs of slides reacted for ER and PR were then rinsed, dehydrated, briefly air dried, coated with Ilford K5.D nuclear track emulsion (Polysciences, Warrington, Pa), exposed for 16–20 days at 4°C and developed in Kodak D-19 developer at 18°C. Some of the paired slides were post-stained in Gill's 3X hematoxylin (Polysciences). Slides were photographed on a Zeiss microscope equipped with planapochromatic lenses at magnifications of 300× and 408× on Kodak Ektar™ 25 film.

The entire serial set for each block was screened to select replicate pairs of slides with the greatest amount of [^{3}H]thymidine labeling. We analyzed the straight glands just beneath the repairing surface of the endometrium and omitted the surface (zone I) and basalis (zone IV). This area was designated zone II/III after the nomenclature of Bartelmez [3] because the distinction between these two zones is not clear during the LFT. Nuclei were scored as [^{3}H]thymidine labeled if at least 10 grains per nucleus were present and as receptor-positive if any detectable immunocytochemical reaction was present. In cases where the reaction was barely discernible, the presence or absence of staining was confirmed with phase contrast optics. Receptor-positive cells included all those with nuclei which exhibited marginal, but perceptible staining, as well as very intensely stained nuclei because we wished to include all cells with any detectable receptor in these measurements. Quantitative analysis of the simultaneous distribution of [^{3}H]thymidine and ER or PR was performed by constructing frequency tables which included the number of [^{3}H]thymidine labeled and/or receptor-positive (ER or PR) nuclei for each block (2 or 3 per animal). The total number of cells evaluated per slide (650–2200) varied according to the total population of epithelial cells within zone II/III. The labeling index (LI), defined as the fraction of [^{3}H]thymidine labeled cells, was extracted from these data. Because the number of stromal

cells in any given section was extremely large, approx. 1000 cells were evaluated per slide.

The percentage of epithelial cells undergoing mitosis in zone II/III was determined by counting the combined number of prophase, metaphase, anaphase and telophase figures in plastic sections of samples of endometrium from each animal (2–3 blocks per animal). Each section contained approx. 750 total epithelial cells in zone II/III.

Statistical analyses

Frequencies of codistribution of [^{3}H]-thymidine and ER or PR for individual blocks were subjected to standard χ^2 analyses. Concentrations of total, cytosolic and nuclear ER, percent receptor distribution, LI and MI were analyzed by ANOVA and pairwise t-tests (Bonferroni probabilities) with Biomedical Data Processing (BMDP) software, University of California Press, Berkeley [19]. The LI data for days 4.5 and 5 were pooled for statistical comparisons. LI and MI data for the 5 animals treated with P for either 1 or 2 days and sampled on day 5 were also pooled for statistical analyses.

RESULTS

PR is present only in stromal cells during the LFT

On day 0 of the LFT (the day the P capsule was removed), PR was detectable in stromal cells of the endometrium, but the glands in zone II/III were PR-negative [Fig. 1(a)]. During the first 4.5 days of P withdrawal in the presence of E_2, PR was evident in stromal cells, but the epithelium was PR-negative until day 5 when some glandular epithelial cells in zone II/III were PR-positive [Fig. 1(b–d)]. There was no difference in the pattern of PR staining between sections of previously frozen blocks and the new samples collected for this study or between fresh frozen blocks and samples incubated in [^{3}H]thymidine before freezing. In the E_2 and P withdrawn, control animals, stromal, but not epithelial PR was detectable, but the intensity of PR staining was significantly reduced [Fig. 3(h)] compared to samples from E_2-maintained animals [Fig. 1(a–d)].

[^{3}H]Thymidine uptake is not correlated with epithelial ER or PR

Zones I and IV. In all animals from all treatment groups, menstruation occurred 2–3 days after the P capsules were removed, and the endometrial surface (zone I) underwent repair on days 4–5. [^{3}H]Thymidine labeled cells were present in the superficial regions of all samples [Fig. 3(e)]. Labeled cells were often difficult to distinguish as epithelial or stromal, but some were clearly epithelial cells in the process of migrating from the mouths of damaged glands to form a new lumenal surface. As we have previously reported [11], these cells were often ER- and PR-negative. This observation confirms the previous suggestion [20] that following menstruation, the endometrial surface can be repaired in the absence of estrogen.

Zone II/III. That E_2 is responsible for the initial rise in epithelial cell division in zone II/III of the endometrium during the LFT was shown by comparing mitotic (MI) and labeling indices (LI) for glandular epithelial cells from the E_2-maintained and E_2 and P withdrawn macaques (Fig. 2). The LI on days 4.5 and 5, and the MI on day 5 are both significantly higher ($P < 0.01$) in the E_2-maintained than the E_2 and P withdrawn group. PR staining was absent from all epithelial nuclei on day 4.5, though many stromal nuclei were ER- and PR-positive in all samples from every animal [Fig. 3(a), (b)]. Thus, the first wave of epithelial DNA synthesis (on day 4.5) in E_2-maintained animals appeared to precede the rise in MI by about 12 h and occurred prior to or simultaneous with the appearance of ER in the epithelium.

By day 5 of P withdrawal, numerous mitoses were visible in glandular epithelial cells of zone II/III. Large numbers of [^{3}H]thymidine labeled nuclei were observed in most glands located in zone II/III, and about one third of all [^{3}H]thymidine-labeled nuclei were ER-negative on day 5 [Fig. 3(c)]. χ-square analyses of the frequency of occurrence of ER and/or [^{3}H]thymidine showed that there was no interaction between the distribution of these two markers. In some samples from experimental animals on day 5 and in two or three blocks from the animal on day 4.5 in which epithelial ER, but not PR was detected, the relationship between the occurrence of [^{3}H]thymidine and ER was *negative* ($P < 0.01$). The remainder of the samples analyzed (all blocks from every animal), showed no significant relationship between the occurrence of ER and [^{3}H]thymidine. In other words there was a tendency for ER and [^{3}H]thymidine to be distributed randomly or in separate cells rather than simultaneously within the same nuclei during the LFT. Data on specimens from control animals also showed no

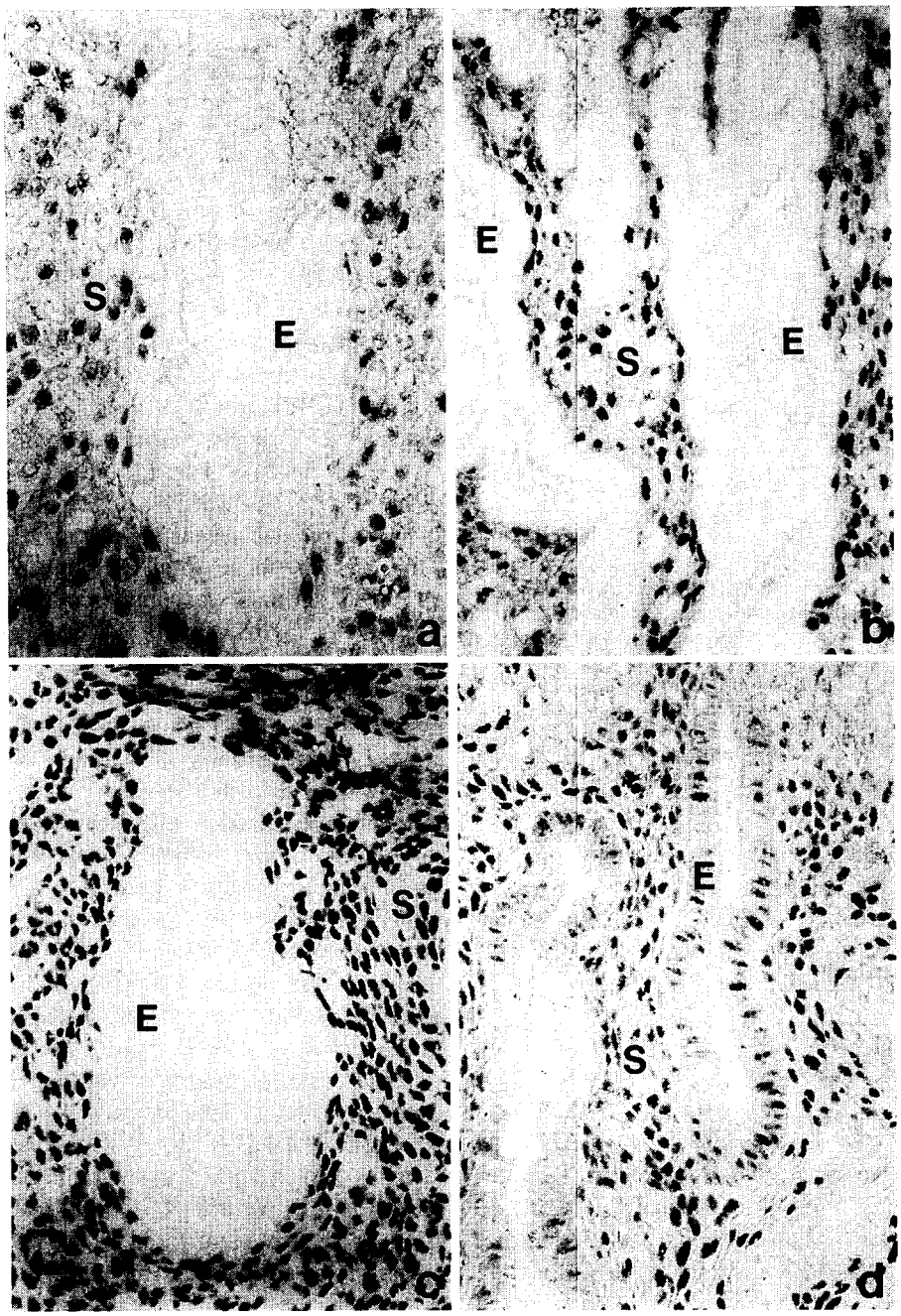

Fig. 1. Immunocytochemistry of PR in endometrial zone II/III during the LFT. Fresh frozen sections of endometria from monkeys treated sequentially with E_2 for 14 days followed by E_2 and P for 14 days (a) and then withdrawn from P for 4 (b), 4.5 (c) or 5 (d) days were stained with 0.5 μg/ml of an antiPR antibody, B-39, as described in the Experimental section. PR was present only in stromal cells (S) until day 5 when some glandular epithelial cells (E) became PR-positive. ×800

direct correlation between ER and [^{3}H]-thymidine except in two cases where it was negative ($P < 0.05$).

Analysis of serial sections also confirmed that on day 5 of the LFT, glands which were PR-positive were always ER-positive as well. In sections reacted for PR that were also prepared for autoradiography, we noted that in certain glands, numerous epithelial nuclei were radioactive and completely PR-negative [Fig. 3(d)]. Regression analyses of the relationship between the fraction of [^{3}H]thymidine-positive versus the fraction PR-positive nuclei indicated that there was no correlation ($R^2 = 0.16$) between the occurrence of [^{3}H]thymidine labeling and epithelial PR. Moreover, χ-square analyses of the

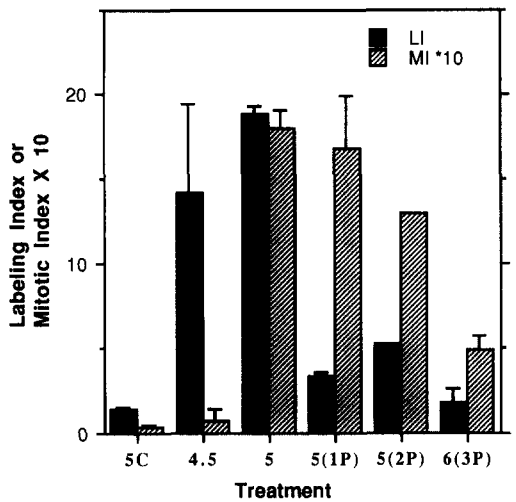

Fig. 2. [^{3}H]Thymidine uptake and mitotic rates in epithelial cells in endometrial zone II/III during the LFT. Data are expressed as the mean ± SEM of the labeling index (LI; shaded bars) or mitotic index (MI; striped bars). All animals were manipulated through one artificial cycle as described in the Experimental section before assignment to one of the following treatment groups: control, day 5 of E_2 and P withdrawal (5C); P withdrawal in the presence of E_2 for 4.5 (4.5) or 5 (5) days; day 5 of P withdrawal with P reinserted for 1 [5(1P)] or 2 [5(2P)] additional days preceding surgery; or day 6 of P withdrawal with P reinsertion for 3 days prior to surgery [6(3P)]. $n = 3$, except for 5(2P), where $n = 2$.

relationship between the presence of PR and [^{3}H]thymidine within the epithelium revealed a significant trend ($P < 0.01$) for these two parameters to be distributed in separate nuclei in all blocks from E_2-maintained animals on day 5 of the LFT.

In the three E_2 and P withdrawn control animals, ER was detected in both stromal and epithelial cells [Fig. 3(g)], but epithelial PR was undetected in serial sections of these same samples. Moreover, stromal PR (present after 14 days of E_2 and P) was actually decreased [Fig. 3(h)]. Serum steroid measurements confirmed that concentrations of E_2 and P were minimal in control animals on day 5 of E_2 and P withdrawal (Table 1).

P-treatment during the LFT inhibits epithelial DNA synthesis but suppresses stromal ER and PR

When a P capsule was reinserted into E_2-maintained animals on day 3 or 4 of the LFT, the LI of zone II/III measured on day 5 was significantly lower ($P < 0.01$) than the LI for E_2-maintained animals not treated with the additional P (Fig. 2). Adding back P for 1, 2 or 3 days during the LFT did not significantly change concentrations of E_2 in serum compared to animals with only E_2 implants (Table 1). Numbers of ER (total, cytosolic and nuclear) in tissues from animals treated with P for either 1 or 2 days on days 4 and 3, respectively, were reduced by approx. 70% as compared to levels in E_2-maintained animals on day 5 ($P < 0.05$; Table 1). The mean total ER concentration in P-treated animals was numerically lower, but not significantly different from control aminals withdrawn from E_2 and P through day 5.

To determine whether the P had suppressed epithelial ER when PR was only detected in the stroma, serial sections of endometria from animals retreated with P before day 5 were immunostained with antiER and antiPR antibodies. In the epithelium, the intensity of the immunocytochemical reaction for ER was indistinguishable in P-treated and E_2-maintained animals. Combined ICC/AR showed that there was no tendency for [^{3}H]thymidine labeled epithelial cells to be preferentially ER-negative or ER-positive after P treatment. Conversely, the additional P treatment resulted in significantly reduced stromal staining for ER on day 5 in zone II/III [Fig. 3(f)]. The exception was stromal cells in zone I which remained moderately ER-positive after P treatment. Although ER was rapidly suppressed in the endometrial stroma after treatment with P, PR staining was intense in the stroma after 1 day of P and remained elevated after 2 days of P exposure. By

Table 1. Effects of P treatment during the LFT on concentrations of ER and serum steroids

	E_2-maintained		Control	P-treated
	Day 4.5	Day 5	Day 5	Day 5+ (1, 2 days P)
Animals (n)	3	5[a]	3	5
Serum E_2 (pg/ml)	125 ± 19	99 ± 12	9 ± 3	133 ± 18
Serum P (ng/ml)	0.19 ± 0.03	0.34 ± 0.08	0.21 ± 0.07	10.3 ± 2.0
Cytosol receptor (fmol/mg DNA)	2481 ± 963	4378 ± 838	3804 ± 1269	1400 ± 262*
Nuclear receptor (fmol/mg DNA)	317 ± 156	876 ± 134	133 ± 16*	225 ± 99
Total receptor (fmol/mg DNA)	2798 ± 1114	5254 ± 941	3937 ± 1284	1625 ± 337*

Data are expressed as mean ± SEM. The number of days of P withdrawal following one artificial cycle is indicated. The number of days of P reinsertion is denoted in parentheses for the appropriate group.

[a]Data from 3 animals appeared previously (Fig. 1; reference [14]).

*Significant differences ($P < 0.05$) in mean receptor values between P-treated or control vs E_2 maintained day 5 groups.

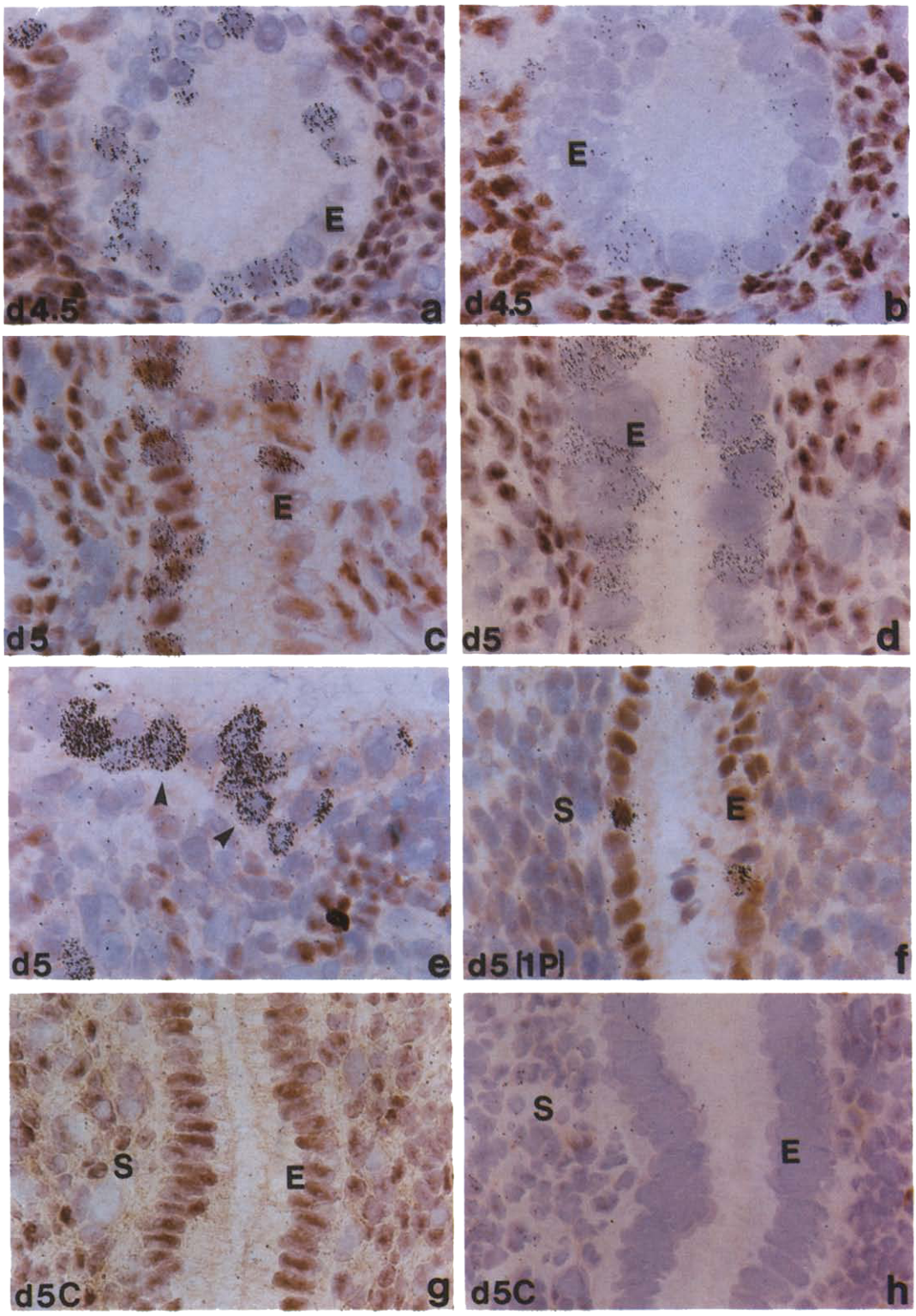

Fig. 3. Combined immunocytochemistry/autoradiography of ER or PR and [^{3}H]thymidine during the LFT. Serial frozen sections of monkey endometria incubated in [^{3}H]thymidine were immunostained with either antiER or antiPR antibodies and then processed for autoradiography as detailed in the Experimental section. Treatment groups are as defined in Fig. 2. (a and b), Day 4.5. Many glandular epithelial cells (E) in zone II/III had [^{3}H]thymidine-labeled nuclei, and most of these were negative for ER (a) and PR (b). (c, d and e), Day 5. Numerous glands were ER-positive, but about one third of all [^{3}H]thymidine-labeled epithelial cells in zone II/III were ER-negative (c). PR was undetectable in serial sections of many ER/[^{3}H]thymidine-positive glands (d). Cells of the repairing surface (zone I) were often labeled with [^{3}H]thymidine (arrowhead), but they were ER-negative (e). (f), Day 5(1P). [^{3}H]Thymidine labeling and stromal (S), but not epithelial, ER staining was decreased [compare (c) and (f)] after adding back P for 1 day. (g and h), Day 5 control. [^{3}H]Thymidine uptake was low in zone II/III, but ER (g) was readily detectable in both stromal and epithelial nuclei though PR (h) was minimally detectable only in the stroma. ×1500

3 days of treatment with P, the stromal reaction for PR was markedly reduced though not absent in zone II/III. Complete suppression of stromal PR by P was not observed, and the intensity of PR staining in the myometrium was not reduced by P. The response of the endometrial basalis (zone IV) to P treatment during the LFT will be reported elsewhere.

The above immunocytochemical observations were confirmed by quantitative analyses of the distributions of ER and PR in zone II/III. About two thirds of all epithelial cells in this region exhibited ER staining on day 5 of the LFT, and about 40% were PR-positive, regardless of whether P was reinserted. The variability in ER and PR immunostaining among blocks from the same animal was high, and the mean percentages of ER- and PR-positive epithelial cells were not different among groups. However, the number of ER-positive stromal cells decreased more than 3-fold after 1 day of P reinsertion and only 10% were ER-positive after 2 or 3 days of P. The percentage of PR-positive stromal cells was unchanged after 1 day of P and decreased to less than 10% of all stromal cells after 3 days of P (Table 2). Because variability in the percentage of receptor-positive cells could obscure actual effects of P, particularly in the epithelium, we normalized these data as the ER to PR ratio for individual serial section pairs from replicates of each block. If P suppression of ER occurred, ER/PR values should be less than one because PR was not as rapidly suppressed as ER. In the epithelium, no single ER/PR datum point for any treatment was less than one, nor was there a decline in the mean ER/PR value after P treatment. In the stroma after one day of P, ER/PR values fell to one third the ratio calculated for samples taken from animals not treated with P. Moreover, the mean ER to PR ratio after one day of P treatment was significantly ($P < 0.01$) lower in stroma than in epithelium (Table 2).

Table 2. Effect of P on cellular distribution of ER and PR detected by ICC

Day	% ER+	% PR+	ER/PR
Epithelium			
5(0P)	66.93 ± 12.56	38.77 ± 5.98	1.81 ± 0.36
5(1P)	62.32 ± 12.27	43.25 ± 10.72	1.53 ± 0.12[a]
5(2P)	61.54 ± 8.02	13.09 ± 6.62	12.13 ± 8.02
6(3P)	55.19 ± 15.02	42.99 ± 18.20	21.74 ± 19.58
Stroma			
5(0P)	81.05 ± 1.79	76.76 ± 1.31	1.06 ± 0.24
5(1P)	24.85 ± 9.89*	82.39 ± 2.23	0.30 ± 0.11[a]
5(2P)	11.97 ± 7.04**	44.07 ± 17.31	0.31 ± 0.22
6(3P)	10.57 ± 3.52**	9.33 ± 2.61**	1.09 ± 0.18

Data are mean percentages or mean ratios of percentages of cells which exhibit immunocytochemically detectable ER or PR ± SEM. The number of days following P withdrawal after one artificial cycle is indicated to the left, and the number in parentheses is the number of days of P treatment immediately prior to hysterectomy. Significantly different mean values for % ER and % PR-positive epithelial or stromal cells in blocks from day 5(0P) specimens vs P-treated samples are denoted by asterisks (*$P < 0.05$) or **$P < 0.01$).

[a]The mean ER/PR ratios for stroma vs epithelium after 1 day of P treatment on day 5 are significantly different, $P < 0.01$.

DISCUSSION

A variety of hypotheses exist to explain the mechanism of estrogen-dependent cell proliferation in normal and neoplastic tissues [21–30]. In the developing rodent reproductive tract, indirect, paracrine mechanisms were postulated to account for observations that underlying stromal cells, but not epithelial (target) cells contained ER when DNA synthesis was initiated in the epithelium [31]. Cunha [32] used reciprocal recombinations of adult epithelia with fetal stroma, and adult stroma with fetal epithelia to show that cell–cell interactions which regulate developmental processes may govern tissue remodeling and hormone action in the adult. Other experiments have shown that stroma can enhance the response of epithelial cells to E_2 either *in vitro* or when transplanted together *in vivo* [33–35]. Thus, there is a growing body of indirect evidence to support the idea that steroid hormone action is dependent on the continued cooperation of the various hormonally responsive cell types within a tissue of the mature organism.

Previously, we suggested that some of the effects of E_2 in the endometrial epithelium of the adult macaque might be mediated by the underlying ER-positive stroma [11]. With ICC/AR we report here that ER and PR are generally not detectable in endometrial epithelial cells during the presumed critical period of E_2 stimulation, and when detected, their distributions are not correlated with the distribution of [^{3}H]-thymidine. Because E_2 and peptide growth factors are thought to act at an early stage in the commitment to DNA replication [36–39], a lag should occur *after* the appearance of epithelial ER before the onset of DNA synthesis to support either a direct or autocrine role for ER in the stimulation of cell proliferation. The negative interaction between [^{3}H]thymidine uptake and the appearance of epithelial PR indicates that separate pathways for E_2 activation of these processes may exist. These pathways could include different cell types, different populations

of epithelial cells or differential timing of these responses within a given cell.

We also found that epithelial DNA synthesis in zone II/III was inhibited by adding back P during the LFT when all PR was detected in the stroma. This P treatment did not prevent the appearance of ER in the epithelium on day 5, even though ER was dramatically reduced in stromal cells on day 5. We know from our previous work [40] that when ER and PR are present in both epithelial and stromal cells, for example after 14 days of E_2 treatment, 1 day of P treatment can dramatically suppress immunocytochemically detectable ER in both endometrial cell types. In this current study, the drop in numbers of ER determined biochemically was correlated with stromal, not epithelial loss of ER, when P treatment began on days 3 or 4 of the LFT. Thus, the lack of P suppression of epithelial ER indicated a lack of direct P action within the epithelium, and the loss of stromal ER marked a direct effect of P in the stroma. The most straightforward interpretation of the inhibitory effect of reinsertion of P during the LFT is that P blocked the initiation of estrogen-dependent DNA synthesis in the epithelium by reducing stromal ER. This is consistent with the view that estrogen-dependent stromal mitogens are the proximal regulators of epithelial DNA synthesis. However, further work is necessary to distinguish between a loss of ER and the potential array of other P-induced changes within the stroma and/or epithelium as causative factors in P inhibition of epithelial proliferation.

The possibility exists that concentrations of ER and PR too small to detect with immunocytochemistry could mediate hormone action. We attempted to discover whether technical difficulties might have resulted in ER loss during the immunocytochemical protocol. Incubation of slices of endometrium *in vitro* with E_2 to maximize the number of occupied ERs and enhance receptor affinity for the nucleus resulted in no change in the overall pattern or intensity of ER immunostaining. Prefixation [41] and freeze substitution [42] improved the retention of ER staining in samples from spayed animals but had little effect on the intensity of ER staining in samples from E_2-maintained animals.

An interesting aspect of this and our previous study is that unlike the rapid proliferative response that occurs following treatment of spayed or immature animals with E_2, the endometrial epithelium remained refractory to E_2 stimulation (as assessed by DNA synthesis, mitotic indices and PR induction) for a relatively lengthy time (4.5–5 days) following P withdrawal. We know P disappears rapidly from the serum because concentrations of P determined in a previous study [11] were less than 1 ng/ml as early as 12 h following P withdrawal. We and others have shown that immunocytochemically detectable ER [40] and PR [43] are present, though minimal, after long term hormone withdrawal. Thus, the initial phase of estrogen action following P withdrawal in the presence of E_2, such as occurs in the natural menstrual cycle, may be a more complex process than E_2 stimulation of tissues withdrawn from hormones for longer periods.

The effect of P on stromal PR observed in this study confirmed recent reports that P suppression of PR is a slower process than P suppression of ER [44, 45]. Data in the human endometrium during the menstrual cycle [46, 47], and studies on the localization of PR in monkeys treated with P in the presence of E_2 or during the natural menstrual cycle [10] suggest that stromal PR is readily detectable even after several days of P exposure. It remains to be determined if concentrations of ER and PR in specific populations of target cells partially rebound from an initial P inhibition or undergo cycles of suppression and recovery.

Significant differences in the proliferative response to E_2 and P occur among the various endometrial zones. Here, we have attempted to define the relationships between ER, PR and estrogen action in the glands of the upper endometrium (zone II/III) following a rapid decline in serum P in the presence of E_2. Our data suggest that interactions between stroma and epithelium may mediate certain aspects of the early proliferative response to E_2 in the glandular epithelium. Whether such interactions continue to operate throughout the follicular phase when ER is distributed in both epithelial and stromal cells deserves consideration.

Acknowledgements—We thank Dr Albyn Jones, Department of Mathematics, Reed College, for help with statistical analyses, Dr Chris Nolan of Abbott Laboratories (North Chicago, Ill.) for the antiER antibody and Dr Geoffrey Greene, Ben May Laboratory, University of Chicago, for the antiPR antibody. We also thank Bev Cole and Kunie Mah for their technical support. This work was supported by NIH grants HD-20380, HD-19182 and P30-HD-181850.

REFERENCES

1. Brenner R. M. and West N. B.: Hormonal regulation of the reproductive tract in female animals. *A. Rev. Physiol.* **37** (1975) 273–302.

2. Hisaw F. L. and Hisaw F. L. Jr: Action of estrogen and progesterone on the reproductive tract of lower primates. In *Sex and Internal Secretions* (Edited by W. C. Young). Williams & Wilkins, Baltimore, 3rd edn, Vol. 1 (1961) pp. 556–589.
3. Bartelmez G. W. with the collaboration of Corner G. W. and Hartman C. G.: Cyclic changes in the endometrium of the rhesus monkey (*Macaca mulatta*). *Contrib. Embryol. (Carnegie Institute, Washington, D.C.)* **34** (1951) 99–144.
4. Padykula H. A., Coles L. G., Okulicz W. C., Rapaport S. I., McCracken J. A., King N. W. Jr, Longcope C. and Kaiserman-Abramof I. R.: The basalis of the primate endometrium: a bifunctional germinal compartment. *Biol. Reprod.* **40** (1989) 681–690.
5. Katzenellenbogen B. S.: Dynamics of steroid hormone receptor action. *A. Rev. Physiol.* **42** (1980) 17–35.
6. Martin L.: Estrogens, anti-estrogens and the regulation of cell proliferation in the female reproductive tract *in vivo*. In *Estrogens in the Environment* (Edited by J. A. McLachlan). Elsevier/North Holland, New York (1980) pp. 103–130.
7. Martin L., Finn C. A. and Trinder G.: DNA synthesis in the endometrium of progesterone-treated mice. *J. Endocr.* **56** (1973) 303–307.
8. West N. B. and Brenner R. M.: Estrogen receptor levels in the oviducts and endometria of cynomolgus macaques during the menstrual cycle. *Biol. Reprod.* **29** (1983) 1303–1312.
9. Brenner R. M., Carlisle K. S., Hess D. L., Sandow B. A. and West N. B.: Morphology of the oviducts and endometria of cynomolgus macaques during the menstrual cycle. *Biol. Reprod.* **29** (1983) 1289–1302.
10. Brenner R. M., McClellan M. C. and West N. B.: Immunocytochemistry of estrogen and progestin receptors in the primate reproductive tract. In *Steroid Receptors in Health and disease* (Edited by V. K. Moudgil). Plenum Publishing, New York (1988) pp. 47–70.
11. McClellan M., West N. B. and Brenner R. M.: Immunocytochemical localization of estrogen receptors in the macaque endometrium during the luteal–follicular transition. *Endocrinology* **119** (1986) 2467–2475.
12. Resko J. A., Norman R. L., Niswender G. D. and Spies H. G.: The relationship between progestins and gonadotropins during the late luteal phase of the menstrual cycle in rhesus monkeys. *Endocrinology* **94** (1974) 128–135.
13. Resko J. A., Ploem J. G. and Stadelman H. L.: Estrogens in fetal and maternal plasma of the rhesus monkey. *Endocrinology* **97** (1975) 425–430.
14. Korenman S. G., Stevens R. H., Carpenter L. A., Robb M., Niswender G. D. and Sherman B. M.: Estradiol radioimmunoassay without chromatography; procedure, validation and normal values. *J. Clin. Endocr. Metab.* **38** (1974) 718–720.
15. Surve A. H., Bacso I., Brinckerhoff J. H. and Kirsch S. J.: Plasma levels of progesterone in pseudopregnant rabbits actively immunized with a progesterone-protein conjugate. *Biol. Reprod.* **15** (1976) 343–349.
16. West N. B. and Brenner R. M.: Progesterone-mediated suppression of estradiol receptors in cynomolgus macaque cervix, endometrium and oviduct during sequential estradiol-progesterone treatment. *J. Steroid Biochem.* **22** (1985) 29–37.
17. McClellan M. C., West N. B., Tacha D. E., Greene G. L. and Brenner R. M.: Immunocytochemical localization of estrogen receptors in the macaque reproductive tract with monoclonal antiestrophilins. *Endocrinology* **114** (1984) 2002–2014.
18. West N. B., Roselli C. E., Resko J. A., Greene G. L. and Brenner R. M.: Estrogen and progestin receptors and aromatase activity in rhesus monkey prostate. *Endocrinology* **123** (1988) 2312–2322.
19. Dixon W. J.: *BMDP Statistical Software*. University of California Press, Berkeley (1985) pp. 105–115.
20. Ferenczy A.: Studies on the cytodynamics of experimental endometrial regeneration in the rabbit. Historadioautography and ultrastructure. *Am. J. Obstet. Gynecol.* **128** (1977) 536–545.
21. Dickson R. B. and Lippman M. E.: Estrogenic regulation of growth and polypeptide growth factor secretion in human breast carcinoma. *Endocr. Rev.* **8**, (1987) 29–43.
22. Lippman M. E., Bates S., Huff K. K., Davidson N. and Dickson R. B.: Estrogens regulate production of specific growth factors in hormone-dependent human breast cancer. *J. Lab. Clin. Med.* **109** (1987) 230–235.
23. Vignon F., Capony F., Chambon M., Freiss G., Garcia M. and Rochefort H.: Autocrine growth stimulation of the MCF7 breast cancer cells by the estrogen-regulated 52 K protein. *Endocrinology* **118** (1986) 1537–1545.
24. Knabbe C., Lippman M. E., Wakefield L. M., Flanders K. C., Kasid A., Derynck R. and Dickson R. B.: Evidence that transforming growth factor-β is a hormonally regulated negative growth factor in human breast cancer cells. *Cell* **48** (1987) 417–428.
25. Stack G. and Gorski J.: Estrogen-stimulated deoxyribonucleic acid synthesis: a ratchet model for the prereplicative period. *Endocrinology* **117** (1985) 2017–2023.
26. Stack G. and Gorski J.: Direct mitogenic effect of estrogen on the prepubertal rat uterus: studies on isolated nuclei. *Endocrinology* **115** (1984) 1141–1150.
27. Mukku V. R. and Stancel G. M.: Regulation of epidermal growth factor receptor by estrogen. *J. Biol. Chem.* **260** (1985) 9820–9824.
28. Soto A. M. and Sonnenschein C.: Cell proliferation of estrogen-sensitive cells: the case for negative control. *Endocr. Rev.* **8** (1987) 44–52.
29. Cunha G. R., Chung L. W. K., Shannon J. M. and Reese B. A.: Stromal-epithelial interactions in sex differentiation. *Biol. Reprod.* **22** (1980) 19–42.
30. Weisz A. and Bresciani F.: Estrogen induces expression of c-fos and c-myc protooncogenes in rat uterus. *Molec. Endocr.* **2** (1988) 816–824.
31. Bigsby R. M. and Cunha G. R.: Estrogen stimulation of deoxyribonucleic acid synthesis in uterine epithelial cells which lack estrogen receptors. *Endocrinology* **119** (1986) 390–396.
32. Cunha G. R., Bigsby R. M., Cooke P. S. and Sugimura Y.: Stromal-epithelial interactions in adult organs. *Cell Diff.* **17** (1985) 137–148.
33. McGrath C. M.: Augmentation of the response of normal mammary epithelial cells to estradiol by mammary stroma. *Cancer Res.* **43** (1983) 1355–1360.
34. Cooke P. S., Fujii D. K., Uchima F-D. A., Sewell L. I., Bern H. A. and Cunha G. R.: Restoration of normal morphology and function in cultured vaginal and uterine epithelia transplanted with stroma *in vivo*. *Endocrinology* **116** (1985) 65 (Abstr. 260).
35. Inaba T., Wiest W. G., Strickler R. C. and Mori J.: Augmentation of the response of mouse uterine epithelial cells to estradiol by uterine stroma. *Endocrinology* **123** (1988) 1253–1258.
36. Epifanova O. I.: Mitotic cycles in estrogen-treated mice: a radioautographic study. *Exp. Cell Res.* **42** (1966) 562–577.
37. Quarmby V. E. and Korach K. S.: The influence of 17β-estradiol on patterns of cell division in the uterus. *Endocrinology* **114** (1984) 694–702.
38. Rozengurt E.: Early signals in the mitogenic response. *Science* **234** (1986) 161–166.
39. Deuel T. F.: Polypeptide growth factors; roles in normal and abnormal cell growth. *A. Rev. Cell Biol.* **3** (1987) 443–492.

40. West N. B., McClellan M. C., Sternfeld M. D. and Brenner R. M.: Immunocytochemistry versus binding assays of the estrogen receptor in the reproductive tract of spayed and hormone-treated macaques. *Endocrinology* **121** (1987) 1789–1800.
41. Yamashita S. and Korach K. S.: A modified immunohistochemical procedure for the detection of estrogen receptor in mouse tissues. *Histochemistry* **90** (1989) 325–330.
42. Brenner R. M., Mah K., West N. B. and McClellan M. C.: An improved immunocytochemical technique for localization of estrogen and progestin receptors in cryostat sections. In *Program of the Eighth International Congress of Histochemistry and Cytochemistry*, Washington D.C. (1988) p. 924 (Abstr.)
43. Okulicz W. C., Savasta A. M., Hoberg L. M. and Longcope C.: Immunofluorescent analysis of estrogen induction of progesterone receptor in the rhesus uterus. *Endocrinology* **125** (1989) 930–934.
44. Wei L. L., Krett N. L., Francis M. D., Gordon D. F., Wood W. M., O'Malley B. W. and Horwitz K. B.: Multiple human progestin agonists and antagonists in breast cancer cells. *Molec. Endocr.* **2** (1988) 62–72.
45. Read L. D., Snider C. E., Miller J. S., Greene G. L. and Katzenellenbogen B. S.: Ligand-modulated regulation of progesterone receptor messenger ribonucleic acid and protein in human breast cancer cell lines. *Molec. Endocr.* **2** (1988) 263–271.
46. Garcia E., Bouchard P., De Brux J., Berdah J., Frydman R., Schaison G., Milgrom E. and Perot-Applanat M.: Use of immunocytochemistry of progesterone and estrogen receptors for endometrial dating. *J. Clin. Endocr. Metab.* **67** (1988) 80–87.
47. Press M. F., Udove J. A. and Greene G. L.: Progesterone receptor distribution in the human endometrium. *Am. J. Path.* **131** (1988) 112–124.