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STEROID RECEPTORS IN THE DEVELOPING AND THE ADULT RABBIT ENDOCERVIX AND IN ENDOCERVICAL EPITHELIAL CELLS ISOLATED BY FLOW CYTOMETRY

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Summary—Immunocytochemistry with monoclonal antibodies H222 and JZB39 was used to study nuclear estrogen (ER) and progesterone (PgR) receptors, respectively, in the cervix during differentiation and in the adult rabbit. The undifferentiated state of the cervix of 2-week-old rabbits correlates with a paucity of immunoreactive nuclear ER, while the epithelium of most of these animals showed moderate immunostaining for the nuclear PgR. The cervical epithelium, stroma and muscle cells of 1-month-old rabbits, showed weak immunostaining for the ER, while staining for PgR remained comparable to that of 2-week-old rabbits. For 2–4-month old rabbits the epithelium was characterized by moderate immunostaining for the nuclear ER and strong immunostaining for the PgR. Strong, heterogeneous immunostaining for nuclear ER and PgR receptors in endocervical epithelial cells from 6-month-old (adult), estrous rabbits suggested there are subpopulations of cells that express differential sensitivity to steroid hormones. In order to characterize such subpopulations, live endocervical epithelial cells were sorted with a flow cytometer on the basis of forward angle light scatter (FSC) and side scatter (SSC) signals which correlated with cell size and secretory granule content, respectively. Secretory cells, as verified by ultrastructural analysis and histochemical staining, expressed the highest FSC and SSC signals and were designated fraction "a". Changes in the hormonal status of the animals altered the intrinsic light scatter properties of fraction "a" cells as follows: maximum FSC and SSC signals were reported for cells from estrous animals; ovariectomy or progesterone-dominance decreased cell size (FCS) and secretory granule content (SSC), while treatment of ovariectomized rabbits with estradiol increased both parameters. When fraction "a" cells from estrous rabbits were incubated with the monoclonal antibodies, two distinct subpopulations of secretory cells were identified by intensity and pattern of nuclear staining for the ER and PgR. Changes in the hormonal status of the animals produced changes in the intensity of nuclear immunostaining, however both cell types remained distinguishable on the basis of immunostain pattern reflecting either permanent or transitory differences in them, and differential hormone sensitivity. The presence of nuclear ER and PgR proteins in these cells confirms their function is bireceptor-mediated.

INTRODUCTION

The endocervix is a steroid target organ where hormone-dependent changes in the viscoelastic properties of mucus glycoprotein(s) play a pivotal role in regulating sperm and bacterial access to the upper reproductive tract [1–3]. In the rabbit, Chilton *et al.* [4, 5] have documented the estrogen dependence of secretory cell morphology and protein glycosylation with ultrastructural studies and metabolic labeling experiments. More recently, Chilton *et al.* [6] have shown that *N*-acetylgalactosaminyl (GalNAc) transferase, the first enzyme in the pathway for O-linked oligosaccharide chain biosynthesis, and oligosaccharyltransferase, the enzyme that catalyzes the first step in the attachment of N-linked oligosaccharide chains to proteins, are regulated by estradiol during development. Furthermore, by manipulating the hormonal milieu of adult rabbits, Chilton *et al.* [6] found that both enzymes are estrogen-dependent and antagonized by progesterone.

The endocervix is comprised of a variety of cell types with potentially different responses to hormones, and receptor assays that require tissue homogenization preclude the localization of receptors in individual cells. As a result, monoclonal antibodies were used to characterize the distribution of nuclear ER and PgR receptors in frozen sections of the cervix during development and in the adult. Then immunocytochemistry

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was coupled to a cell isolation strategy in order to demonstrate heterogeneity in the receptor content of subpopulations of secretory epithelial cells in the adult. Flow cytometry was used to rapidly sort large numbers of live mucus-secreting cells on the basis of forward angle light scatter (cell size) and side scatter (secretory granule content) signals. Hormone-dependent changes in the light scatter profiles of these epithelial cells, as well as hormone-dependent changes in their receptor content are reported here. Differential immunostaining for receptors in subpopulations of secretory cells is concordant with the idea that steroids differentially regulate epithelial cell function.

METHODS

Reagents and buffers

Reagents were obtained from the following sources: mouse monoclonal anti-epithelial keratins (AE1 and AE3) and FITC conjugated goat antimouse Fab were purchased from ICN ImmunoBiologicals (Lisle, Ill.). Rat monoclonal antibodies to estrogen (H222) and progesterone (JZB39) receptors were provided by Abbott Labs, Diagnostic Division (Abbott Park, Ill.) and G. Greene, Ben May Labs for Cancer Research, University of Chicago (Chicago, Ill.). Peroxidase Rat IgG Vectastain ABC Kit was purchased from Vector Laboratories Inc. (Burlingame, Calif.). Haemaccel, a plasma replacement manufactured by Hoechst–Roussel Pharmaceuticals Inc., was provided by Paul W. Eldridge, Manager, Flow Cytometry Facility, Department of Cell Biology-Anatomy, Texas Tech Health Sciences Center (Lubbock, Tex.).

Phosphate-buffered saline (PBS) consisted of 20 mM sodium phosphate, pH 7.5 and 0.9% sodium chloride. Solution A consisted of 0.1% BSA in calcium–magnesium-free Tissue Culture Medium purchased from Gibco (Grand Island, N.Y.). Solution B was a fixative for living cells composed of 45% Iscove's Modified Dulbecco's Medium (Sigma Chemical Co., St Louis, Mo.), 5% Haemaccel and 50% methanol [7].

Animal treatments

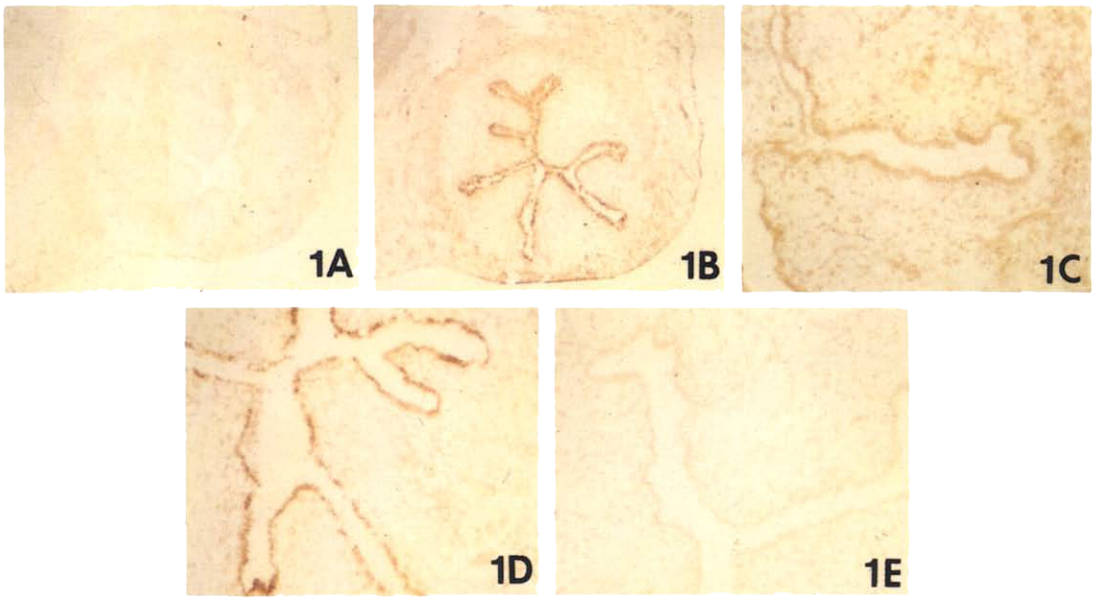
Juvenile New Zealand white rabbits of the following ages were used for experimentation: 2 weeks ($n = 14$), 1 month ($n = 6$), 2 months ($n = 4$) and 4 months ($n = 4$). Adult New Zealand White rabbits (6 months of age) were housed for 3 weeks to insure against reflex ovulation resulting in pseudopregnancy prior to experimentation. All studies were conducted in accord with the NIH Guide for the Care and Use of Laboratory Animals. Rabbits ($n = 35$) with mature ovarian follicles at the time of laparotomy were designated estrous. Endocervical folds (>90% epithelium) from these animals were used in 28 flow cytometry experiments. A second group of estrous animals ($n = 6$) was bilaterally ovariectomized through a mid-ventral incision and maintained for a minimum of 16 weeks. Four of these long-term ovariectomized (LTOVX) rabbits remained untreated and were used in two flow cytometry experiments. The other two animals received 20 injections of 17β-estradiol (5 μg/12 h) and were killed 12 h after the last injection for use in two flow cytometry experiments. Ethanol:corn oil (1:99) was used as the vehicle, and steroid was injected s.c. in a volume of 100 μl.

A third group of estrous rabbits ($n = 15$) was made pseudopregnant (PSP) with an ear vein injection of 20 IU hCG (40 μl), followed by cervical stimulation with a glass rod. These PSP animals were killed 5 days after the injection. Pseudopregnancy was verified by the presence of 9 ± 1 corpora lutea/animal. These animals were used in 15 flow cytometry experiments.

Flow cytometry, immunostaining and electron microscopy

Preparation of cell suspensions from endocervical folds was as previously described [4, 5, 8]. Briefly, endocervical folds (>90% epithelium) were pronase-digested, mechanically dissociated and filtered to remove tissue fragments. Monodispersed cells were sorted on the basis of forward-angle light scatter (FSC) and side scatter (SSC) with a Facstar Plus flow cytometer (Becton Dickinson, Mountain View, Calif.) utilizing the 488-nm line from an argon laser at 250 mW. Analysis windows were adjusted to accommodate changes in the distribution of cell populations. FITC fluorescence was spectrally detected with a 530/30 nM filter. Data from 10,000 cells/fraction for each experiment were stored in a list mode format and analyzed using Facstar Plus Research Software.

For immunostaining, cells ($1–7.5 \times 10^5$) were incubated on ice in solution B for 15 min, collected by centrifugation and washed with solution A. Cells were then resuspended in solution A with and without (controls) monoclonal anti-epithelial keratins AE1 and AE3 (1:25) and incubated on ice for 30 min. Cells were collected by centrifugation, washed twice



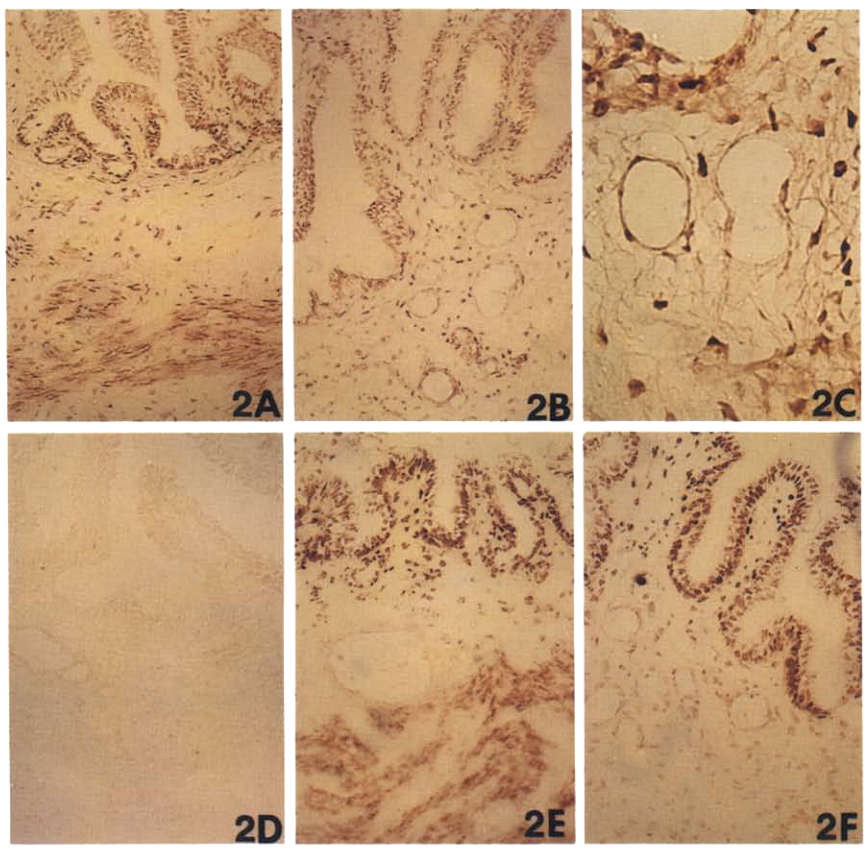


Fig. 1. Nuclear ER is absent from endocervical cells of 2-week-old rabbits (A) and nuclear PgR is localized in the epithelial cells (B) of these whole tissue sections. Immunostaining for the nuclear ER is increased in intensity in the cervix of 2-month-old rabbits (C), and immunostaining for the PgR remains strong in the epithelium (D). Immunostaining is negligible when non-immune serum is substituted for the monoclonal antibody (E). A–B = ×25, C–E = ×100

Fig. 2. Immunostaining for the nuclear ER in whole tissue section from an estrous rabbit (A) and endothelial cells of capillaries (B and C). Immunostaining is undetectable when non-immune serum is substituted for the monoclonal antibody (D). Immunostaining for the nuclear PgR in whole tissue section from an estrous rabbit (E). Immunostaining is absent from the endothelial cells of capillaries (F). A–B, D–F = ×80, C = ×320

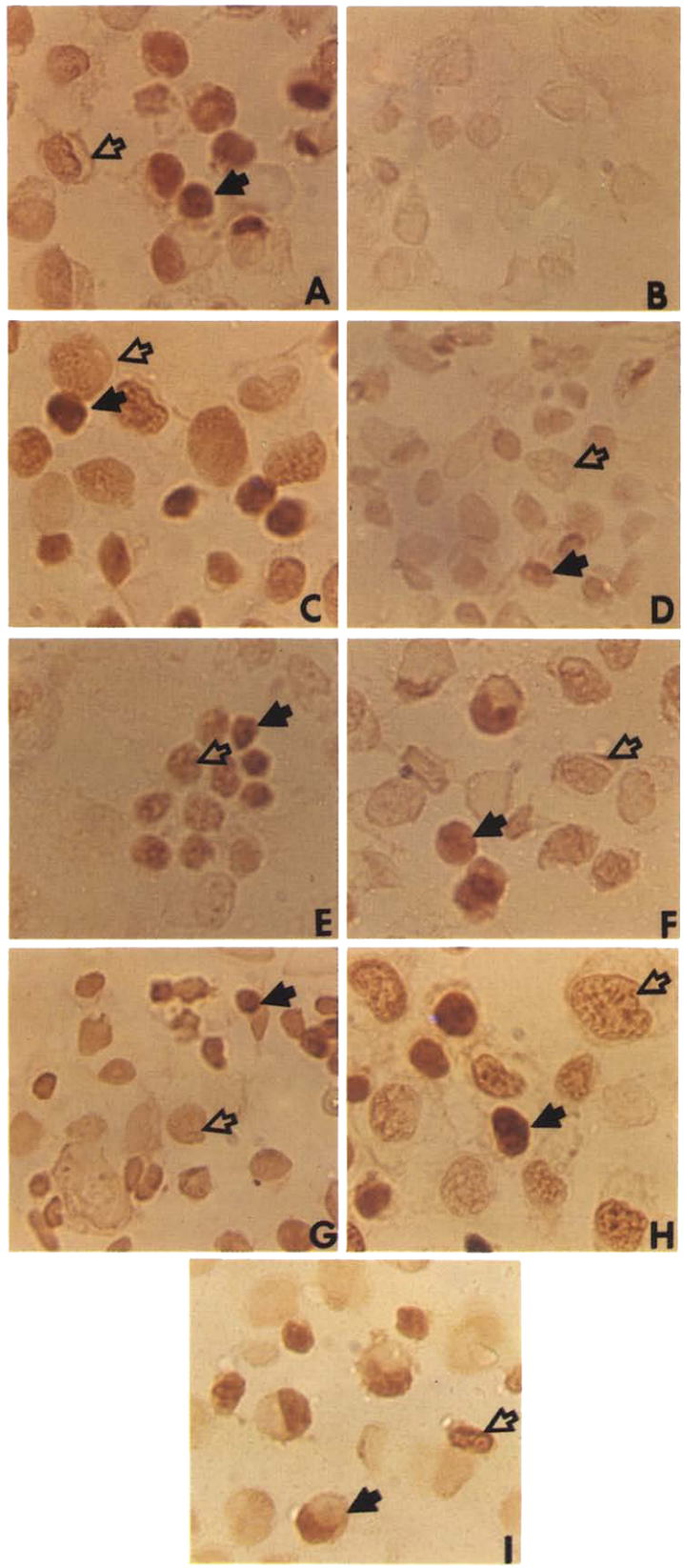


Fig. 5. Two distinct secretory epithelial cell types are identified by intensity and pattern of nuclear staining for the ER in estrous (A) animals. Immunostaining is more intense for type A cells (solid arrow) compared with type B cells (open arrow). Staining is negligible when non-immune serum is substituted for the monoclonal antibody (B). Note the stain pattern for the nuclear PgR in both type A (solid arrow) and type B (open arrow) cells from estrous animals (C) closely resembles the pattern characteristic of the ER. Nuclear staining for the ER is reduced in cells from LTOVX (D) rabbits, and is restored to estrous control levels when LTOVX rabbits are treated with estradiol (E). A modest decrease in the nuclear immunostaining for ER is evident for cells from PSP animals (F). Intensity and pattern of nuclear staining for PgR, is similar to that reported for the nuclear ER, in LTOVX (G), LTOVX + E_2 (H) and PSP (I) animals. ×600

with solution A, resuspended in solution A containing FITC conjugated goat anti-mouse Fab (1:16) and incubated on ice for 30 min. Cells were again collected by centrifugation, washed as described and fluorescence was analyzed with the Facstar Plus to determine the percentage of epithelial cells.

Aliquots of cells ($1–2 \times 10^5$) were pelleted (600 rpm, 10 min) onto microscope slides in a Shandon Cytospin 2 cytocentrifuge (Shandon Southern Instruments Inc., Sewickley, Pa). For histological studies, cells were fixed with 1% paraformaldehyde and stained for the presence of glycoproteins by a modified periodic acid-Schiff (PAS) technique and quantitated as previously described [4, 5]. For immunocytochemical localization of ER and PgR, cytocentrifuged cells were fixed with 1% paraformaldehyde, permeabilized with 85% ethanol, rinsed with PBS and preincubated with normal rabbit serum (NRS) for 30 min at room temperature. Cells were subsequently incubated with H222 (7.2 μg/ml) or JZB39 (9.2 μg/ml) overnight in a moist chamber at 4°C as described by West *et al.* [19]. Sixteen to 24 h later, cells were rinsed with PBS, preincubated with NRS, and then incubated with reagents from the Vectastain kit according to the manufacturer's instructions. Controls included the substitution of NRS for primary monoclonal antibody.

Fresh, frozen cervical tissue from estrous rabbits, was sectioned (3 μm) in a cryostat. Sections were mounted onto uncoated glass slides and stored at −80°C. Sections were subsequently warmed to room temperature, fixed immediately (1% paraformaldehyde) and immunostained as described above. Two individuals judged the slides for staining intensity and ranked the results as strong (+ + +), moderate (+ +), weak (+) or absent (−).

For transmission electron microscopy, cell pellets were prepared as previously described [4, 5, 8] and viewed with a Hitachi H600 electron microscope operated at 75 kV.

RIA procedures

Serum progesterone and 17β-estradiol levels were determined by Dr David Hess, Oregon Regional Primate Research Center (Beaverton, Oreg.) as previously described [6]. The recovery of tritiated estradiol in the total assay was 78.1, and the sensitivity of this assay was less than 1.0 pg/ml. The total recovery of tritiated progesterone was 85.9%, and the sensitivity of this assay was 10 pg/assay tube. The intrassay coefficients of variation were 9–10% for both assays. For each sample steroid concentrations were estimated for sample volumes of 100 and 400 μl, and the results were averaged if they were between 10–90% of the limits of the standard curve. Standard parametric statistics and Student's *t*-test were used in the evaluation of changes in serum steroid titers.

RESULTS

The undifferentiated state of the endocervical canal of 2-week-old rabbits [6] correlates with a paucity of immunoreactive nuclear ER as summarized in Table 1. Of the 14 animals tested, immunoreactive ER was at or below the limit of detection in the epithelium of 10 animals and the stroma and muscle cells of 8 animals [Fig. 1(A)]. In contrast, the epithelium of most of these animals showed moderate immunostaining for the nuclear PgR, while the stroma and muscle cells were weakly immunostained [Fig. 1(B)]. As shown in Table 1, the epithelium, stroma and muscle cells of 1-month-old rabbits showed weak immunostaining for the ER, and immunostaining for the PgR remained comparable to 2-week-old rabbits. For 2-month-old rabbits, immunostaining for the ER was weak for stroma and muscle cells and moderate for epithelial cells [Fig. 1(C)]. Weak immunostaining for ER was apparent in the endothelial cells of some blood vessels. Immunostaining for the PgR was strong in the epithelium and weak to moderate in the stroma and muscle cells [Fig. 1(D)]. Immunostaining for ER and PgR in the cervices of 4-month-old rabbits was comparable to 2-month-old animals, except immunostaining for the PgR in the muscle cells was strong.

As shown in Fig. 2(A), strong, heterogeneous immunostaining for the nuclear ER was localized in the endocervical epithelium of

Table 1. Localization of steroid receptors in the cervices of juvenile rabbits

Age	*n*	ER	PgR	Region of cervix
2-weeks	14	−	+ +	Epithelium
		−	+	Stroma
		−	+	Muscle cells
1-month	6	+	+ +	Epithelium
		+	+	Stroma
		+	+	Muscle cells
2-months	4	+ +	+ + +	Epithelium
		+	+ +	Stroma
		+	+ +	Muscle cells
4-months	4	+ +	+ + +	Epithelium
		+	+ +	Stroma
		+	+ + +	Muscle cells

6-month-old, estrous rabbits. Strong immunostaining was apparent for stromal cells and muscle cells of the fibromuscular wall, as well as muscle cells (tunica media) of the endocervical arteries. Moderate immunostaining was present in the nuclei of endothelial cells of capillaries [Fig. 2(B)], especially those located in close proximity to the epithelium [Fig. 2(C)]. Specificity of immunostaining was confirmed by negligible staining in control tissue sections [Fig. 2(D)]. Strong immunostaining for the nuclear PgR was prominent in epithelial cells [Fig. 2(E)], and as noted for the ER, the nuclei of some cells appeared more immunopositive than others. Moderate immunostaining for PgR was localized in the nuclei of stromal and muscle cells. Weak immunostaining was detectable in the muscle cells of some arteries and absent or below the limit of detection in endothelial cells of capillaries [Fig. 2(F)].

Because of the marked heterogeneity in immunostaining, epithelial cells from estrous animals were sorted with a flow cytometer on the basis of forward angle light scatter (FSC) and side scatter (SSC) signals which correlated with cell size and the content of cytoplasmic secretory granules, respectively. As shown in Fig. 3(A), cells from estrous animals with the highest FSC and SSC signals were designated fraction "a". This fraction represented $35 \pm 1\%$ of the total and was comprised of non-vacuolated (type A) and vacuolated (type B) cells engorged with heterogeneous secretory granules [Fig. 4(A) and (B)]. The glycoprotein content of the secretory granules was verified by the fact that $85 \pm 2\%$ of the cells in fraction "a" gave a positive stain response to periodic acid-Schiff [PAS] reagent, a 2-fold enrichment over the original aliquot of unsorted cells. Flow cytometric analysis of cells immunostained with

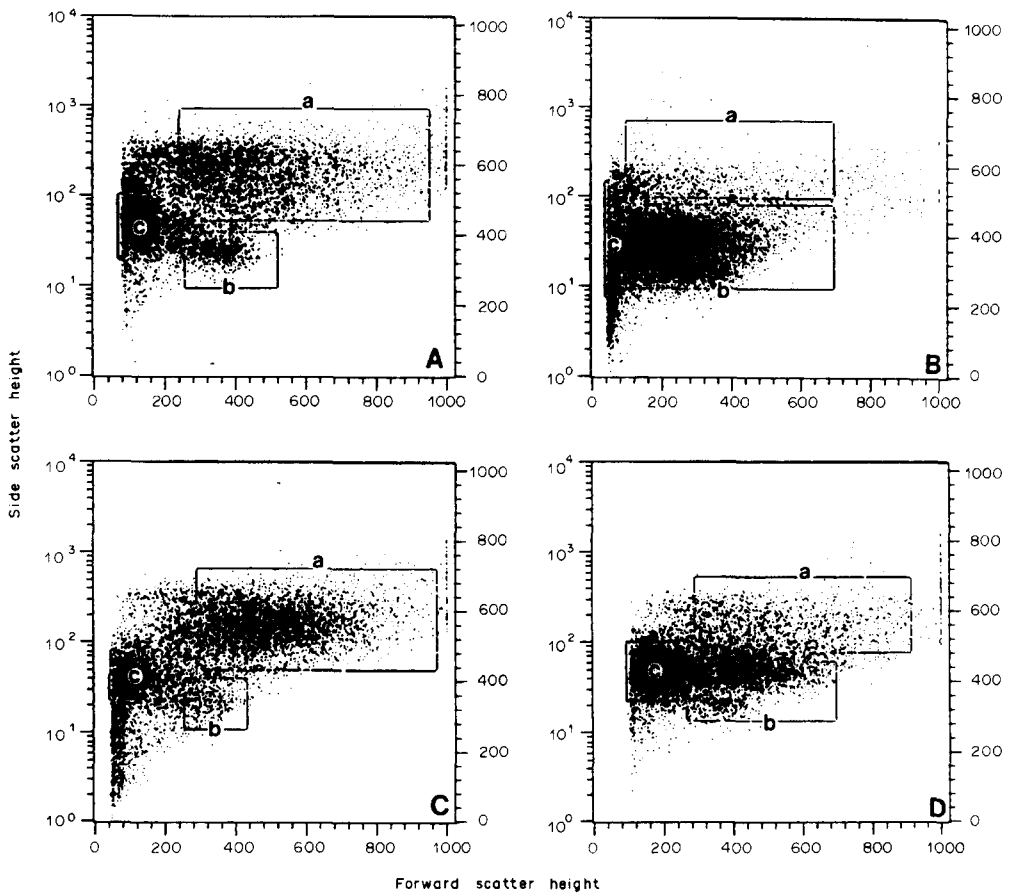


Fig. 3. Light scatter profiles for endocervical cells from estrous (A), LTOVX (B), LTOVX + E_2 (C) and PSP (D) animals. Fraction "a" contains cells with the highest FSC and SSC signals. The cells in this fraction are PAS-positive, secretory cells quantitated following cytocentrifugation and staining with periodic acid-Schiff reagent. Fraction "b" cells are PAS-negative cells and secretory cells with a reduced number of secretory granules; and, fraction "c" contains erythrocytes and leukocytes. Note the changes in the light scatter profiles of fraction "a" secretory cells in response to steroid hormones that alter their size and internal complexity (number of secretory granules).

anti-epithelial keratins confirmed the epithelial identity of 73 ± 3% of the fraction "a" cells and eliminated the possibility that stromal cells were actually being evaluated. Values resulting from flow cytometric analysis were consistently lower than values for PAS-staining because fragile cells were lost during the fixation and staining processes that preceed flow cytometric evaluation. A second population of PAS-negative cells (81%) designated fraction "b" was devoid of secretory granules and therefore had reduced FSC and SSC signals. These cells represented 8 ± 1% of the total cell suspension and only 42 ± 6% of them were epithelial cells. Fraction "c" contained erythrocytes and leukocytes, i.e. cells with the lowest FSC and SSC signals.

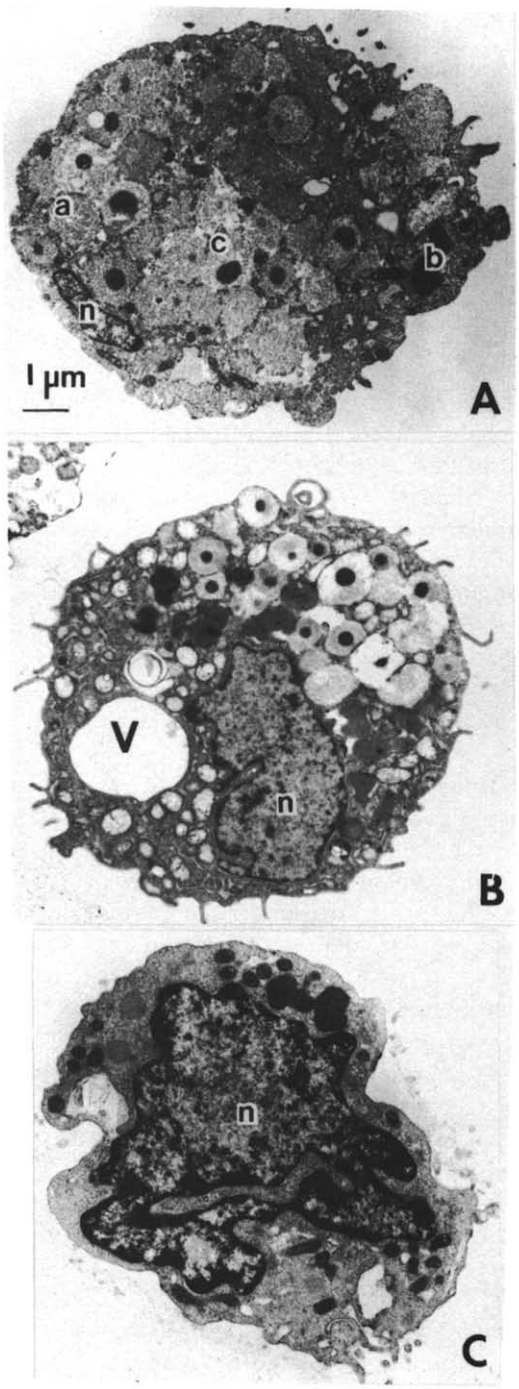


Fig. 4. Electron micrographs of fraction "a" secretory epithelial cells. The type A cell (A) from estrous rabbits is engorged with heterogeneous mucous granules designated electron-lucent (a), electron-dense (b) and mixed density with an inner dense core (c). The typical type B cell (B) from estrous rabbits contains all three types of secretory granules plus one or more empty cytoplasmic vacuoles (V). Note the absence of granules in the fraction "a" secretory cell (C) from an LTOVX animal. Cell nuclei are designated n. × 7500

As shown in Fig. 5(A), when fraction "a" cells from estrous rabbits were incubated with the monoclonal antibody H222, two distinct populations of secretory cells were identified by intensity and pattern of nuclear staining for the ER. Type A cells were characterized by uniform monochromatic stain superimposed on a punctate distribution of stain, while type B cells were characterized by a punctate distribution of stain only. Nuclear staining for type A cells was more intense than for type B cells. Immunostaining was negligible in the cytoplasm of the secretory cells. Immunostaining was also negligible for controls [Fig. 5(B)]. Serum titers of estradiol and progesterone for these animals were 13.5 ± 1.8 pg/ml and 0.36 ± 0.10 ng/ml, respectively.

As shown in Fig. 5(C), when fraction "a" cells from estrous rabbits were incubated with the monoclonal antibody JZB39, the stain pattern for nuclear PgR in both type A and type B cells closely resembled the pattern characteristic of the ER. Immunostaining for the nuclear PgR in type A cells was more intense than for type B cells. Specific immunostaining in the cytoplasm of the secretory cells was negligible.

In order to determine the effect of the endocrine state of the animal on receptor immunostaining in fraction "a" cells, it was necessary to first determine whether the endocrine state of the animal modulated the FSC and SSC signals of secretory cells potentially altering the cellular composition of the fraction. Thus, endocervical cells from animals in each treatment group were analyzed by flow cytometry. As shown in Fig. 3(B), fraction "a" cells from LTOVX animals represented 14 ± 3% of the total cell suspension. The negligible PAS-stain response of these cells was correlated with a paucity of secretory granules [Fig. 4(C)]. Flow cytometric analysis of fraction "a" confirmed that 77% of these cells were epithelial. The reduction in the

intracellular compliment of secretory granules resulted in reduced FSC and SSC signals such that epithelial cells were redistributed to fraction "b" from fraction "a". Approximately 79% of the fraction "b" cells were epithelial, a 2-fold increase over the fraction "b" value for estrous controls. Estrogen administration (5 μg 17β-estradiol/animal/12 h for 10 days) to LTOVX rabbits resulted in the restoration of fraction "a", secretory cells with FSC and SSC signals [Fig. 3(C)] comparable to estrous control animals. Here, fraction "a" comprised $44 \pm 2\%$ of the total, 97% of the cells were PAS-positive and 80% of the cells were epithelial in origin.

The total number of PAS-positive cells was reduced from $48 \pm 3\%$ for estrous animals to $17 \pm 3\%$ for progesterone dominated, PSP animals in the original aliquot of dispersed cells. As shown in Fig. 3(D), fraction "a" from PSP animals was enriched 4–5-fold for PAS-positive cells ($77 \pm 4\%$) despite a reduction in cell number, i.e. fraction "a" cells represented only $24 \pm 3\%$ of the total population, and $70 \pm 2\%$ of the cells were epithelial in origin. The decrease in FSC and SSC signals resulted in a shift of cells from fraction "a" to "b" such that fraction "b" now represents $15 \pm 3\%$ of the total and contains $34 \pm 4\%$ eithelial cells. This hormone-dependent change in the FSC and SSC signals precisely correlates with a decrease in the average granule volume of secretory cells [10].

Following cell sorting, fraction "a" cells from animals in all treatment groups were immunostained for ER and PgR. First, cells from LTOVX rabbits were incubated with the monoclonal antibody H222 and compared with estrous controls. Individual variability in the overall intensity of nucler staining was correlated with a measurable overlap in the range of serum estradiol values for LTOVX (3–9 pg/ml) and estrous (7–17.5 pg/ml) rabbits. In general, stain intensity was reduced dramatically for cells from LTOVX animals concomitant with a 3-fold decrease in the serum titer of estradiol (5.4 ± 0.9 pg/ml). However, type A and type B cells could be distinguished by the residual pattern of nuclear staining for the ER [Fig. 5(D)]. When LTOVX rabbits were treated with estradiol for 10 days, the intensity of nuclear immunostaining [Fig. 5(E)] and the serum titers of estradiol (17.3 ± 1.0 pg/ml) were restored to estrous control levels.

For PSP rabbits there was a modest decrease in the immunostaining with H222 for the nuclear ER [Fig. 5(F)], but type A and type B cells could be distinguished. The concentration of serum estradiol for these animals (15.7 ± 1.5 pg/ml) did not vary significantly from that of estrous controls, but the concentration of serum progesterone was increased 27-fold to 9.8 ± 1.5 ng/ml.

When cells from LTOVX rabbits were incubated with the monoclonal antibody JZB39 and compared with estrous controls, a modest reduction in the intensity of nuclear staining [Fig. 5(G)] was noted. However both type A and type B cells could be identified. When LTOVX rabbits were treated with estradiol for 10 days, the degree of immunostaining in type A and type B cells [Fig. 5(H)] was comparable to that of estrous controls. Immunostaining in the cytoplasm of secretory cells was negligible.

For PSP rabbits compared with estrous control animals, a decrease in the intensity of immunostaining for the nuclear PgR occurred, however the characteristic pattern of nuclear immunostaining persisted such that type A and type B cells could be distinguished from each other [Fig. 5(I)].

DISCUSSION

We have previously documented the undifferentiated state of the lower reproductive tract of juvenile, female rabbits [6, 11]. For 2-week-old rabbits, cross-sections of endocervical tissue were indistinguishable from cross-sections of uterine tissue, and serum estradiol and uterine ER levels were reduced ($P < 0.01$) compared with adult values [6]. Moreover, an increase in steroid–receptor dissociation was correlated with a reduced rate of activation of steroid–receptor complexes [6], thus limiting the biological effect of estrogen on this undifferentiated tissue. Whole uteri (+ cervices) were homogenized for these assays which precluded the localization of receptor in specific regions of the tissue, and the cervix accounted for less than 15% of the total wet weight of tissue. Therefore, immunocytochemistry was used to determine the amount and distribution of nuclear ER and PgR in the developing cervix. These studies were facilitated by the availability of well characterized monoclonal antibodies that have been used to detect receptors in various target organs from the rabbit [12–14]. Amplification of the histochemical reaction with these monoclonal antibodies was achieved with the Biotin/Avidin system [15].

The paucity of immunostaining for the ER in cervices from 2-week-old animals and the localization of PgR in the epithelial cells is dramatically different from what has been reported for the uteri of the immature rodents [16–21]. In general, ER is detected in the stroma of the fetal genital tract (uterus, cervix and vagina), prior to its expression in the epithelial cells; and ER is detectable much earlier in gestation than PgR. For guinea-pigs uterine ER levels increase during fetal development but decrease after birth [22]. Conversely, the number of uterine receptors reaches a maximum on day 10 for neonatal rats [19] demonstrating that significant variability exists between animal models. The most important finding from our studies is that the expression of ER and PgR preceeds the expression of the estrogen-dependent/progesterone-antagonized enzymes that regulate glycoprotein biosynthesis [6] in the juvenile rabbit cervix. Moreover, the increase in the amount of immunoreactive ER in the epithelium of 6-month-old rabbits compared to 4-month-old rabbits, parallels the dramatic increase in the size of epithelial cells and the volume of their secretory granules [6].

Current theories on the role of estradiol in regulating the viscoelastic properties of cervical mucus include: (1) a direct effect of estradiol on glycoprotein biosynthesis by secretory endocervical epithelial cells [6]; (2) a direct effect of estradiol on endocervical capillary permeability which would change the concentration of mucin by altering its level of hydration [23]; (3) an indirect effect of estrogen through induced mediators, so call mucinotropic agents, such as prostaglandins, basic polypeptides and histamine, which are present in serum and stroma and promote the release of secretory glycoproteins by epithelial cells [24]. The presence of ER in secretory endocervical cells, as verified by biochemical analysis [25] and immunocytochemistry, confirms these cells are targets for the direct action of estradiol thereby legitimizing theory (1) above. The presence of PgR in these cells indicates that some cellular functions are bireceptor mediated. However, the distribution of PgR in whole tissue from the rabbit cervix differs from that reported by Perrot-Applanat *et al.* [26]. In their studies endocervical epithelial cells from diethylstilbestrol-treated, immature rabbits contained little or no immunostainable PgR. Several major differences distinguish the two studies not least of which is the fact that these authors used different antibodies plus fixed and paraffin-embedded tissue. We used frozen sections in this study because Press and Greene [14] reported the strongest and most consistent staining was observed with this condition. The presence of ER in the stroma and endothelial cells of capillaries strengthens theories (2) and (3). Clearly, the presence of ER in the endothelial cells of the endocervix distinguishes it from the rabbit uterus where immunoreactivity in capillary endothelial cells was undetectable with H222 [13], and underscores the suggestion by Hass *et al.* [23] that estrogen acts directly to alter capillary permeability in the endocervix.

Marked heterogeneity in immunostaining for cells of the same type has been reported by others [12, 13, 26] for whole tissue sections. As a result, the isolation of subpopulations of cells by flow cytometry offers a new, sophisticated approach to the study of steroid hormone receptor localization. Here, for the first time, viable endocervical epithelial cells were analyzed and sorted on the basis of their intrinsic light scatter properties, hormone-dependent changes in the light scatter profiles of secretory cells were documented, and differential immunostaining for nuclear ER and PgR in subpopulations of secretory cells was demonstrated conclusively. This work was expedited by the fact that a suitable cell dissociation technique had been developed [4, 5]. The combination of enzymatic and mechanical dissociation resulted in high yields of viable, non-clumped cells. In addition, cells were not preferentially lost as a result of the dissociation procedure.

When endocervical epithelial cells were analyzed and sorted on the basis of their intrinsic light scatter properties, forward angle light scatter (FSC) and side scatter (SSC) signals were consistently reproducible for cells from estrous animals. Ovariectomy or progesterone-dominance decreased cell size (FCS) and secretory granule content (SSC) of glycoprotein containing cells, while treatment of LTOVX rabbits with 17β-estradiol increased both cell size (FCS) and secretory granule content (SSC). Cells in fraction "c", i.e. non-epithelial cells with the lowest FCS and SSC signals, were unaffected by the hormonal milieu.

In previous publications [8, 10, 25] two subpopulations of secretory cells were designated type I and type II cells. However, in order to avoid confusion with the type I and type II forms of the estrogen receptor, we have substituted the designations type A for type I cells and

type B for type II cells. Both cell types contained secretory granules and both were actively involved in glycoprotein biosynthesis, as determined by metabolic labeling experiments. The only significant morphological difference between the two cell types was the presence of one or more empty cytoplasmic vacuoles in type B cells [10]. After an extensive morphological study, Odor and Blandau [27] confirmed our observation and concluded that type A and B cells are the same secretory cell type. Using small populations of secretory cells isolated by unit gravity sedimentation and a single saturating dose assay for ER, Chilton [25] demonstrated that type A and type B cells account for 90% of the total endocervical epithelial ER. However, the concentration of ER in type A cells was approx. 50% higher than the value for type B cells from estrous rabbits, suggesting a significant difference in the hormonal sensitivity of the two secretory cell types.

Now, these studies have been extended by the use of a rat anti-ER antibody to visualize the differential intensity and pattern of nuclear immunostaining for the ER in type A and type B cells. The first evidence for PgR in isolated populations of rabbit endocervical epithelial cells has also been provided by the use of a rat anti-PgR antibody. Differential intensity and pattern of immunostaining for the PgR were also evident for type A and type B cells. Type A cells consistently displayed the highest intensity of immunostaining for both ER and PgR. Differential immunostaining for the nuclear ER and PgR in secretory endocervical epithelial cells suggests differential hormone sensitivity of the secretory cells and could represent a potentially powerful component of endocrine regulation. At the very least, differential immunostaining reflects either permanent or transitory differences between type A and type B cells not heretofore detected by morphological evaluation.

Nuclear immunostaining for the ER and PgR persisted in the endocervical epithelial cells of LTOVX rabbits. Perhaps this argues in favor of the hypothesis that unliganded receptor resides in the nucleus [9, 14, 26], binds to its DNA response element and inhibits transcription [28]. Alternatively, perhaps steroid-occupied receptor resides in the nucleus as a result of low but persistent levels of adrenal (?) steroid (cf. Table 1). Press *et al.* [29] demonstrated that the estrogen receptor was localized in the euchromatin of target cells, and Perrot-Applanat *et al.* [30] found that progesterone receptor distribution in the nucleus varied according to hormonal treatment. Thus, ultrastructural analysis of immunocytochemical data should help to resolve the question of whether the distinctive nuclear immunostain patterns reported for the type A and type B cells represent the association of receptor with specific nuclear structures.

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