Immunohistochemical localization of the oestrogen-responsive 110 kDa and 74 kDa polypeptides and complement component C3 in the rat genital tract after oestrogen treatment and during the oestrous cycle

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In previous studies we showed that two oestrogen-responsive rat uterine secretory polypeptides of molecular mass 110 and 74 kDa share sequence similarities with the α and β chains of rat complement component C3. Using polyclonal antibodies specific to the 110 kDa polypeptide, the 74 kDa polypeptide and rat serum C3, we studied the localization of these proteins in the rat genital tract using an indirect immunoperoxidase technique. The results showed that in oestradiol-treated rats immunostaining for the three antigens was heterogeneously distributed in the epithelia of the endometrium, cervix and vagina and that the intensity of staining was greater after oestrogen treatment than in controls. In oestradiol-treated immature rats, the immunoreactivity for the 110 kDa and 74 kDa polypeptides was greater in the endometrial and vaginal epithelia than in the cervical epithelium, whereas immunoreactivity for C3 was greatest in the vaginal epithelium. In cyclic rats, staining by all three antibodies was seen only during pro-oestrus and oestrus, during which stages the immunostaining for the 110 kDa and 74 kDa polypeptides was detected only in the endometrial epithelium, whereas immunostaining for C3 was also found in the epithelia of the cervix and vagina. In general, consecutive sections of these tissues revealed a close correlation between the immunostaining for the 110 kDa and 74 kDa polypeptides and C3. However, there were some sections that showed clear differences in staining, suggesting that more than one C3-related protein species was detected in the female rat reproductive tract.

Introduction

The female genital tract undergoes cyclic morphological, biochemical and physiological alterations that are hormone dependent (Mueller *et al.*, 1958). However, there is embryological and histological evidence that indicates that the responses to ovarian hormones in the different tissues of the genital tract are not the same (Katzman *et al.*, 1971). Numerous studies have documented significant changes associated with the synthesis and secretion of proteins in rabbit (Beier, 1982), rat (Kuivanen and DeSombre, 1985; Komm *et al.*, 1985, 1986; Lyttle *et al.*, 1987; Takeda *et al.*, 1988) and mouse uterus (Katz *et al.*, 1980; Korach *et al.*, 1981; Finlay *et al.*, 1982; Teng *et al.*, 1986) in response to steroid stimulation. However, only a few studies have compared these changes with the changes that occur in the expression of proteins in the cervix and vagina in response to steroid hormones (Katzman *et al.*, 1971; Malnick *et al.*, 1983).

We identified two uterine secretory polypeptides the molecular mass of which under reducing conditions are 110 kDa and 74 kDa (Kuivanen and DeSombre, 1985). Synthesis of these proteins is stimulated with oestradiol in immature and mature ovariectomized rats (Kuivanen and DeSombre, 1985),

is inhibited by exogenous progesterone (Kuivanen and DeSombre, unpublished observations), and occurs only during the oestradiol-dominant pro-oestrous and oestrous stages of the oestrous cycle (Kuivanen and DeSombre, unpublished observations). We reported that these polypeptides are disulfide-linked subunits of a protein with a molecular mass under non-reducing conditions of 180 kDa (Kuivanen *et al.*, 1989) and that their amino acid sequence is similar to the α and β subunits of rat complement component C3 (Kuivanen *et al.*, 1989). On the basis of these results, we proposed that the oestrogen-responsive uterine 180 kDa protein is related to C3. Lyttle *et al.* (1987) identified two similar uterine secretory proteins, with molecular masses of 115 and 65 kDa. They also observed that the proteins are synthesized in the oestradiol-treated ovariectomized mature (Komm *et al.*, 1985) and immature (Komm *et al.*, 1986) rats and are subunits of a 180 kDa protein (Lyttle *et al.*, 1987). On the basis of nucleotide sequence analysis of a LE-1 cDNA encoding the 3' end of the 180 kDa protein, Sundstrom *et al.* (1989) concluded that the 180 kDa protein is C3.

It is well known that there are components of the immune system of the female rat reproductive tract that are responsive to hormone modulation. Immunoglobulin A (IgA), secretory component and immunoglobulin G (IgG) are regulated by steroid hormones in the rat endometrium, cervix and vagina

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(Sullivan and Wira, 1984; Wira and Sullivan, 1985; Parr and Parr, 1985a, 1989). Sundstrom *et al.* (1989, 1990) demonstrated increased synthesis of C3 and an increase in the concentration of its mRNA in uterine epithelial cells of immature rats after treatment with oestradiol and after treatment with the anti-oestrogens tamoxifen, LY 117018 and LY 156758. Brown *et al.* (1990) showed that the C3 is synthesized in the uterus during the oestrous cycle and that progesterone inhibits the oestrogen-stimulated synthesis of this protein.

Together these studies show steroid regulation of the expression of the 110 kDa and 74 kDa polypeptides and C3 in the rat uterus. However, little is known about the presence of these proteins in the lower genital tract of rats. We used rabbit polyclonal antibodies specific for the denatured 110 kDa and 74 kDa polypeptides (Kuivanen *et al.*, 1989) and a sheep polyclonal antibody to rat serum C3, to compare the distribution of the oestradiol-responsive 110 kDa and 74 kDa polypeptides and C3 in the rat uterus, cervix and vagina before and after treatment with exogenous oestrogen and during the oestrous cycle. With this immunohistochemical approach, we localized these proteins in specific types of cell of the rat genital tract and characterized the similarities and differences in their localization following stimulation by oestrogen.

Materials and Methods

Animals

Immature (20-day-old) female Sprague-Dawley derived rats (Sasco Inc., Omaha, NE) were injected s.c. once a day for 3 days with either 1 µg oestradiol in 0.1 ml 10% ethanol in saline (oestradiol-treated immature) or 0.1 ml vehicle alone (control immature). Mature female Sprague-Dawley derived rats were bilaterally ovariectomized under ether anaesthesia at 81 days of age and one week later received three daily s.c. injections of either 5 µg oestradiol in 0.1 ml vehicle (oestradiol-treated mature) or vehicle alone (control mature). All injections were at 24 h intervals. The animals were killed by decapitation 6 h after the third injection, at which time the rate of synthesis of the 74 and 110 kDa polypeptides was maximal (Kuivanen and DeSombre, unpublished observations). For the oestrous cycle study, mature animals were housed in separate cages in air-conditioned facilities maintained on a 12 h light:12 h dark cycle; food and water were available *ad libitum*. Vaginal smears were examined at the same time each day for three weeks. The stage of the cycle was identified on the basis of the cellular composition of the vaginal smear (Nicholas, 1962), and only those animals showing three consecutive four-day cycles were used.

Tissue collection and fixation

The tissues of the genital tract (uterus, cervix, vagina), and liver were surgically removed and immediately immersed in Bouin's fixative for 8 h. All tissues were dehydrated in a graded ethanol series, cleared in xylene, embedded in paraffin wax, and cut into 4–6 µm thick sections.

Antibodies

The rabbit polyclonal antibodies B₁₉ and C₉ are monospecific for the 110 kDa and 74 kDa polypeptides, respectively, show no crossreactivity with one another, and are immunoreactive with the native 180 kDa uterine protein under non-reducing conditions (Kuivanen *et al.*, 1989). The sheep polyclonal anti-rat C3 antibody (ICN ImmunoBiological, Lisle, IL) is immunoreactive with rat serum C3 and the native 180 kDa uterine protein under non-reducing conditions and is not immunoreactive with either the 110 kDa or 74 kDa polypeptide subunit under reducing conditions (Kuivanen *et al.*, 1989).

Immunocytochemical procedure

Deparaffinized sections were immunostained with the streptavidin-biotin-peroxidase complex system (Zymed Laboratories Inc., South San Francisco, CA), following the steps described for indirect peroxidase techniques (Hsu *et al.*, 1981). All incubations were carried out in a moist chamber, and all dilutions and washes were done with PBS (0.05 mol phosphate-buffered saline l⁻¹, pH 7.5). After dewaxing, rehydration and washing, the sections were immersed in 0.3% H₂O₂ in methanol for 30 min to inhibit endogenous peroxidase activity. Those sections that were to be immunostained with the rabbit primary antibodies were incubated sequentially with (i) 3% normal goat serum (NGS) (Zymed Laboratories) for 30 min at room temperature to block nonspecific binding of the secondary antibody, (ii) the primary rabbit polyclonal antibody B₁₉ or C₉ (1:1000 dilution) for 48 h at 4°C, (iii) biotinylated goat anti-rabbit IgG (Zymed Laboratories) (1:100 dilution) for 30 min at room temperature, (iv) horseradish peroxidase-conjugated streptavidin (Zymed Laboratories) (1:100 dilution) for 30 min at room temperature, and (v) 0.35% 3,3-diaminobenzidine tetrahydrochloride (DAB, Sigma Chemical Co., St Louis, MO) in 0.05 mol Tris buffer l⁻¹ pH 7.5 containing 0.03% H₂O₂ for 5 min at room temperature (Ciocca and Björcke, 1986). Negative controls were sections incubated with 3% non-immune rabbit serum (NRS) or 3% NGS. For C3 immunostaining, the sections were first incubated with 3% normal donkey serum (NDS) (Chemicon International, Inc., El Segundo, CA) for 30 min at room temperature and then with the primary sheep polyclonal anti-rat C3 antibody at an optimal dilution of 1:200 for 24 h at 4°C. The sections were then incubated with affinity-purified biotinylated donkey anti-sheep IgG (Chemicon International) (1:100 dilution) and then with peroxidase-streptavidin complex (1:100 dilution), each for 30 min at room temperature. Peroxidase activity was revealed by DAB staining as described above. Negative controls were sections incubated with 3% NDS or immunoabsorbed sheep anti-rat C3 antiserum, which was prepared by first diluting the antiserum 1:200 and then incubating it overnight at 4°C with an excess of normal rat serum. Positive controls were sections of liver incubated with the primary antibody. After colour development, some sections were counterstained with haematoxylin-eosin before being washed, dehydrated, cleared in xylene and mounted with PRO-TEXX (Baxter Laboratories, Deerfield, IL). The samples were observed and photographed with a Nikon photomicroscope, using phase contrast optics whenever required. Positive

Table 1. Distribution of 110 kDa, 74 kDa and C3 proteins in tissues of the genital tract of oestradiol-treated immature rats

Tissue	Staining intensity ^a		
	110 kDa	74 kDa	C3
Endometrium			
Luminal epithelium	++	+++	++
Glandular epithelium	-/+	-/+	-/+
Stromal cells	-	-/+	-
Cervical epithelium	+/+++	+/++++	+/++
Vaginal epithelium	++	++	+++

^aIntensity of immunostaining is graded: - negative, + weak, ++ moderate, +++ strong.

immunostaining was semiquantitatively graded on an arbitrary three-point scale: weak (+), moderate (++) and strong (+++) staining.

Results

In oestradiol-treated immature rats, immunoreactivity for the three proteins was detected in endometrial, cervical and vaginal epithelia (Table 1). In adult cyclic rats, immunoreactivity for the three proteins was detected only at pro-oestrus and oestrus. In the cyclic rat, the immunoreactivity for the 110 and 74 kDa polypeptides was restricted to the endometrium, whereas immunostaining for C3 was also present in the cervix and vagina (Table 2).

Localization of the 110 kDa and 74 kDa polypeptides and C3 in rat endometrium

Very weak immunoreactivity for the 110 kDa and 74 kDa polypeptides was observed in the luminal epithelium of control immature rats and no staining was observed in the glandular epithelial cells (Fig. 1a). No immunostaining for C3 was observed in the endometrium of control immature rats (Fig. 1b). However, the luminal epithelium of oestradiol-treated immature rats displayed strong immunoreactivity for the 74 kDa polypeptide (Fig. 1c) and moderate immunostaining for the 110 kDa polypeptide (Fig. 1d). In the glandular epithelial cells of these endometria, there was weak immunoreactivity for both the 110 kDa and 74 kDa polypeptides (Figs 1c, d). The endometria of some oestradiol-treated immature rats showed a few stromal cells that were immunoreactive for the 74 kDa polypeptide (data not shown). In all of the consecutive sections of the endometrium studied, we observed that the luminal and glandular epithelial cells that stained for the 74 kDa polypeptide also stained for the 110 kDa polypeptide (Figs 1c, d). In the endometria of oestradiol-treated immature rats, the spectrum of immunostaining for C3 varied from moderate in the apical and basal cytoplasm of the luminal epithelial cells to weak in the glandular epithelium (Fig. 1e). Most of the consecutive sections of the endometrium studied showed that the luminal and glandular epithelial cells that exhibited immunoreaction for C3 (Fig. 1e) also displayed immunostaining for the 74 kDa polypeptide (Fig. 1c) and 110 kDa polypeptide (Fig. 1d). However, there were some consecutive sections that immunostained for

the 74 kDa polypeptide (Fig. 1f) that showed little or no immunoreactivity for C3 (Fig. 1g). The pattern of staining with all three antibodies was heterogeneous in intensity and distribution (Figs 1c-f). Although the whole cytoplasm of the luminal and glandular epithelial cells showed immunoreactivity for the three proteins, the staining of the apical cytoplasm was greater than that of the basal region (Figs 1c-f). Sections incubated with NRS were unstained for the 110 kDa and 74 kDa polypeptides (data not shown) and sections incubated with the sheep anti-rat C3 antiserum pre-absorbed with rat serum remained unstained for C3 (Fig. 1h).

No immunostaining was observed for these proteins in the endometrial epithelia of control mature rats, but in oestradiol-treated mature rats, moderate to weak immunoreactivity for the three proteins was detected (data not shown).

Localization of the 110 kDa and 74 kDa polypeptides and C3 in rat cervix and vagina

In the epithelia of the cervix and vagina from control immature and control mature animals, no immunostaining for the 74 kDa, 110 kDa polypeptides and C3 was observed (data not shown). In oestradiol-treated immature rats, immunoreactivity for these proteins was detected in the epithelial cells of the intermediate and superficial layers of the cervix (Fig. 2a-c) and in the parabasal cells of the vagina (Figs 2d-f). The cytoplasm of these cells displayed strong to moderate immunostaining for the 74 kDa and 110 kDa polypeptides (Figs 2a-e), while the spectrum of C3 immunostaining varied from weak in the stratified epithelium of the cervix (Fig. 2c) to strong in the vaginal epithelium (Fig. 2f). In oestradiol-treated mature rats, the cervical and vaginal epithelia, at best, showed only weak staining for the 74 kDa and 110 kDa polypeptides (Fig. 2g, immunostaining for the 74 kDa polypeptide in the vaginal epithelium), whereas staining for C3 varied from moderate to strong (Fig. 2h, vaginal epithelium). Like the endometrium, the epithelia of the cervix and vagina displayed a heterogeneous pattern of immunostaining for the three proteins. Most of the consecutive sections studied showed that the epithelial cells that exhibited immunoreaction for C3 (Fig. 2c, f, h) also displayed immunostaining for the 74 kDa (Fig. 2a, d, g) and 110 kDa polypeptides (Fig. 2b, e). Comparison of the relative intensities of staining for the three proteins revealed that the cervical epithelia of oestradiol-treated immature rats showed greater staining for the 110 and 74 kDa polypeptides than for C3 (compare Fig. 2a and b with c). However, the vaginal epithelia of oestradiol-treated mature rats showed greater staining for C3 than for either the 110 kDa or 74 kDa polypeptide (compare Fig. 2h and g). As in the endometrium, immunostaining in the cervical and vaginal epithelia was specific, as they were unstained after incubation with either NRS or sheep anti-rat C3 antiserum pre-absorbed with rat serum (data not shown).

Localization of the 110 and 74 kDa polypeptides and C3 in mature cyclic rats

During pro-oestrus and oestrus, immunostaining for the 74 and 110 kDa polypeptides in the endometrium was heterogeneous and varied in intensity from moderate in the luminal epithelial cells to weak in the glandular epithelium (Fig. 3a and

Table 2. Distribution of 110 kDa, 74 kDa and C3 proteins in tissues of the rat genital tract during the oestrous cycle

Stage of oestrous cycle	Tissue	Staining intensity ^a		
		110 kDa	74 kDa	C3
Pro-oestrus and oestrus	Endometrium			
	Luminal epithelium	+/+	+/+	+/+
	Glandular epithelium	-/+	+	-/+
	Stroma	-	-	-
Metoestrus and dioestrus	Cervical epithelium	-	-	+/+
	Endometrium	-	-	-
	Cervical epithelium	-	-	-
	Vaginal epithelium	-	-	-

^aIntensity of immunostaining is graded: - negative, + weak, ++ moderate.

b, immunostaining for 74 and 110 kDa polypeptides, respectively, in the pro-oestrous endometrium). Immunostaining for C3 was moderate to weak in the luminal and glandular epithelial cells of the endometrium (Fig. 3c, pro-oestrous endometrium). In contrast to the uterus, no immunostaining for the 110 and 74 kDa polypeptides was observed in the stratified epithelia of the cervix and vagina (Fig. 3d, immunostaining for 74 kDa polypeptide in the pro-oestrous vagina). In the cervical epithelium, staining for C3 was weak and localized only in the superficial cell layer, whereas in the vaginal epithelium it was moderate and localized in the intermediate and superficial cell layers (Fig. 3e, pro-oestrous vagina).

During metoestrus and dioestrus, no immunostaining for 110 kDa, 74 kDa or C3 was observed in the endometrium or in the cervical and vaginal epithelia (data not shown).

Discussion

In this study, we used polyclonal antibodies specific for the 110 and 74 kDa denatured polypeptide subunits of the uterine 180 kDa protein and a polyclonal antibody to native rat serum C3 to show that these proteins are localized in the epithelial cells of the rat uterus, cervix and vagina, and that these proteins are regulated by oestrogen in all of these epithelia. Our results expand the work of Sundstrom *et al.* (1989), who suggested that the oestrogen-regulated expression of C3 is specific only to the uterus.

Comparing the responses of the different uterine cell types to oestrogen treatment, we consistently observed the greatest immunoreactivity for the 110 and 74 kDa polypeptides in the endometrial luminal epithelial cells of immature rats after oestrogen treatment. These results are consistent with earlier isotope incorporation studies that showed that cellular proteins of comparable molecular mass are synthesized *in vitro* by explants of uterine tissue from oestradiol-treated immature rats (Kuivanen and DeSombre, 1985; Lyttle *et al.*, 1987). Like the 110 and 74 kDa polypeptides, immunoreactivity for C3 in the endometrium was mainly localized in the luminal epithelial cells, confirming the earlier findings of Sundstrom *et al.* (1989). Within the epithelial cell, the greatest intensity of staining for

the three proteins was in the apical region of the cytoplasm, supporting the hypothesis that these proteins are secretory products of the epithelial cells (Kuivanen and DeSombre, 1985; Sundstrom *et al.*, 1989). Other oestrogen-regulated uterine proteins, i.e. rat uterine secretory protein-1 (Takeda *et al.*, 1988) and the cat uterine secretory protein (Murray and Verhage, 1985), have also been localized in the apical cytoplasm of luminal and glandular epithelial cells. We also found evidence of immunostaining for the 74 kDa polypeptide in a few stromal cells of oestradiol-treated immature rats. However, the source of the polypeptide in these cells is not yet known.

In the cyclic rat, immunostaining for the three proteins was evident in the endometrial epithelium only during pro-oestrus and oestrus, when endogenous concentrations of oestradiol are high (Brown-Grant *et al.*, 1970), and was not evident during metoestrus and dioestrus. These results correlated well with earlier *in vitro* labelling studies that showed that secretory proteins of comparable molecular mass were synthesized by uterine explants from rats at pro-oestrus and oestrus, but not by explants from rats at metoestrus and dioestrus (Brown *et al.*, 1990; Kuivanen and DeSombre, unpublished observations).

Taken together, the evidence suggests that the uterine antigen reactive with the three antibodies is synthesized locally in the epithelial cells of the endometrium. In addition to the *in vitro* incorporation studies cited above, immunoprecipitation studies revealed that *in vitro* labelled proteins of comparable molecular mass immunoprecipitated from the media of explants of uterine tissue from oestradiol-treated immature rats (Kuivanen *et al.*, 1989; Sundstrom *et al.*, 1989). In addition, *in situ* hybridization studies and northern blot analysis localized C3 mRNA in the luminal epithelial cells of oestradiol-treated immature rats (Sundstrom *et al.*, 1989; Brown *et al.*, 1990). However, our data cannot exclude the possibility that there is also transudation of serum C3 into the luminal epithelial cells. We showed that oestrogen increased the secretion of specific serum proteins in the uterus of immature rats (Kuivanen and DeSombre, 1985). Others have reported that the presence of the serum proteins albumin, peroxidase and plasminogen activator in the oestrogen-stimulated uterus of immature rats is the result of transudation of serum proteins and the infiltration of uterine eosinophils and plasma cells (Peterson and Spaziani, 1971; King *et al.*, 1981;

Fig. 1. Immunocytochemical localization of 110 kDa, 74 kDa and C3 proteins in immature rat endometrium. (a, b) Sections of uterine endometrium from control immature rats showing weak, heterogeneous immunostaining for 74 kDa polypeptide in the luminal epithelium (a, arrows) and no immunostaining for C3 (b, arrows). The sections are counter-stained with haematoxylin. (c–e) Consecutive sections of endometrium from oestradiol-treated immature rats showing strong immunostaining for 74 kDa polypeptide (c, arrows), moderate staining for 110-kDa polypeptide (d, arrows), and moderate staining for C3 (e, arrows) in the apical cytoplasm of the luminal epithelium. All three proteins showed slight immunostaining in the glandular epithelium. The staining is heterogeneous for the three proteins. (f, g) Consecutive sections of endometrium from oestradiol-treated immature rats showing strong, heterogeneous immunostaining for the 74 kDa polypeptide (f, arrows) and very weak immunostaining for C3 (g, arrows) in the luminal epithelium. (h) A consecutive section of endometrium of oestradiol-treated immature rats was treated with sheep anti-rat C3 antiserum pre-absorbed with normal rat serum and shows no immunostaining for C3 in the luminal epithelium (arrow). Bars represent 1 μ m.

Fig. 2. Immunocytochemical localization of 110 kDa, 74 kDa and C3 proteins in cervix and vagina of oestradiol-treated rats. (a–c) Consecutive sections of cervix from oestradiol-treated immature rats showing strong immunostaining for (a) 74 kDa polypeptide, (b) moderate to strong staining for 110 kDa polypeptide, and (c) weak immunoreactivity for C3 in the intermediate and superficial cell layers. Note that the staining is heterogeneous for the three proteins. (d–f) Consecutive sections of vagina from oestrogen-treated immature rats displaying moderate, heterogeneous immunostaining for (d) 74 kDa and (e) 110 kDa polypeptides and (f) strong immunoreactivity for C3 in the parabasal, intermediate, and superficial cell layers. (g and h) Consecutive sections of vagina from oestrogen-treated mature rats displaying weak immunostaining for (g) 74 kDa polypeptide and (h) strong immunostaining for C3 in the parabasal and intermediate cell layers. Bars represent 1 μ m.

Kneiffel *et al.*, 1982). It may be that transudation of serum C3 into the oestrogen-stimulated uterus is superimposed upon the local synthesis of the 180 kDa uterine protein.

In contrast to the uterus, little is known about oestrogen-responsive proteins in the cervix and vagina of rats (Katzman *et al.*, 1971; Malnick *et al.*, 1983). Here, we report immunostaining

Fig. 3. Immunocytochemical localization of 110 kDa, 74 kDa and C3 proteins in endometrium and vagina of mature cyclic rats. (a–c) Consecutive sections of endometrium from rats at pro-oestrus showing weak to moderate immunostaining for (a) 74 kDa polypeptide, (b) 110 kDa polypeptide, and (c) C3 in the luminal and glandular epithelia. (d) and (e) Consecutive sections of vagina from rats at pro-oestrus displaying (d) no immunoreactivity for 74 kDa polypeptide and (e) moderate immunoreactivity for C3 in the intermediate and superficial cell layers. Bars represent 1 μ m.

for the 110 and 74 kDa polypeptides in the epithelial cells of the cervix and vagina of immature and mature rats after oestrogen treatment. However, immunostaining for these polypeptides was not detected in these stratified epithelia during pro-oestrus or oestrus. Their absence in cyclic rats could be due to low or undetectable amounts of the antigen in these epithelia, as a result of lack of synthesis or as a result of their active secretion. In contrast, immunostaining for C3 was evident in the stratified epithelia of the vagina and cervix in immature and mature rats after oestrogen treatment and in the mature cyclic rat during pro-oestrus and oestrus. We show here that the immunoreaction of C3 in these epithelia changes during the oestrous cycle.

The morphology of the rat vagina and cervix, in particular, reveals an absence of glands associated with the mucosa and has generally been considered inappropriate for the existence of a secretory immune system (Vaerman and Ferin, 1974). However, the secretory component and IgA have been detected in the rat vaginal epithelium during pro-oestrus (Parr and Parr, 1989). They are thought to be derived mainly from serum (Parr and Parr, 1985a, 1989). Although the origin of the C3 in the cervical and vaginal epithelia in the study reported here is not known, there is evidence suggesting that this protein is synthesized locally in these squamous epithelial cells. Earlier isotope incorporation studies revealed synthesis of a 180 kDa secretory protein by explants of the cervix from oestradiol-treated immature rats *in vitro* (Komm *et al.*, 1986; Lyttle *et al.*, 1987).

However, these workers questioned the source of this protein and suspected that the explants may have been contaminated with uterine tissue. In humans, C3 has been detected immunocytochemically in the cervical mucosa (Schumacher, 1980), where its concentration changes during the menstrual cycle (Schumacher, 1980). Synthesis of C3 has been observed in cultures of human vaginal mucosa (Lai *et al.*, 1973), as well as in cultures of human keratinocytes and in a carcinoma cell line (Basset-Séguin *et al.*, 1990). Whether the presence of C3 in the rat cervical and vaginal epithelia reported here is the result of local synthesis or transudation needs to be determined.

In the cervix and vagina, the immunoreactivity of the three proteins was localized mainly in the upper epithelial cell layers, which are devoid of oestrogen receptors (Leroy *et al.*, 1969; Stumpf, 1969). Consistent with our observations, heparin sulfate proteoglycan has also been localized in the superficial cell layers of the mouse vaginal epithelium following oestrogen stimulation (Hayashi *et al.*, 1988). This raises the question of whether oestrogen acts directly via its receptor to regulate the synthesis of these proteins in the superficial cell layer or whether oestrogen acts indirectly via a paracrine pathway in which stromally derived mediators influence epithelial proliferation and differentiation (Cunha *et al.*, 1985) and the expression of these proteins. Sundstrom *et al.* (1989, 1990) and Brown *et al.* (1990) have correlated increased synthesis of C3 in the oestrogen-stimulated rat uterus with a corresponding increased concentration of its mRNA, suggesting that, in the rat uterus,

hormonal regulation of the expression of C3 is at the level of transcription.

Immunostaining with the three antibodies in this study was greater in the tissues of the genital tract of oestradiol-treated immature rats than in the same tissues from oestradiol-treated mature rats or cyclic rats. This difference may be related to differences in the responsiveness of the cells as a function of their state of differentiation or their concentration of oestrogen receptors. The concentration of oestrogen receptors in target tissues varies as a function of the age of the animal (Clark and Gorski, 1970). During development of the rat uterus, the synthesis of the induced protein (IP), for example, is maximal between 10–15 days of age (Katzenellenbogen *et al.*, 1980), at which age the concentration of oestrogen binding sites per cell is highest (Clark and Gorski, 1970). Similarly, the induction of the 57 kDa oestrogen-responsive protein and $\gamma\gamma$ enolase are observed only in uteri from oestradiol-treated immature rats (Mairesse and Galand, 1982).

The 110 and 74 kDa polypeptides are the disulfide-linked subunits of a 180 kDa uterine protein and share a high degree of sequence similarity with the α and β subunits of rat serum C3 (Kuivanen *et al.*, 1989; Sundstrom *et al.*, 1989). The antibodies used in the study reported here were polyclonal and could not discriminate between two very similar proteins. We therefore hypothesized that the three antibodies detect the same antigen and show very similar immunoreactivities with the tissues of the rat reproductive tract. Indeed, we observed consecutive sections of the endometrial, cervical and vaginal epithelia that stained for both the 110 and the 74 kDa polypeptide, consistent with the contention that the polypeptides are the subunits of the same protein. Furthermore, we observed that, in general, those cells that stained for the two polypeptides also stained for C3, consistent with the similarity between the uterine 180 kDa and rat serum C3 proteins. However, we also observed that staining for the 74 kDa polypeptide was consistently greater than staining for the 110 kDa polypeptide, even though they are subunits in equivalent proportion in the same protein. This difference in relative staining intensities could be explained by differences in the relative sensitivities of the antibodies or by differences in concentration or stability of the respective antigenic subunit in the tissue. Moreover, we observed that some of the consecutive sections of endometrial and cervical epithelia that stained for the 110 and 74 kDa polypeptides showed almost no staining for C3. These staining patterns were observed not only in oestradiol-treated immature rats, but also in oestradiol-treated mature rats. We also observed that in the oestradiol-treated rats immunoreactivity for the 110 and 74 kDa polypeptides was greatest in the endometrial epithelia, while staining for C3 was greatest in the vaginal epithelium. These different staining patterns were even more pronounced in the cervical and vaginal epithelia during pro-oestrus and oestrus, when immunostaining for the 110 and 74 kDa polypeptides was absent, whereas immunoreactivity for C3 was clearly present. The reason for these different staining patterns is not presently understood, although there are several possible explanations.

One explanation is that the antibodies detected differences in the packaging or degree of post-translational processing, for example glycosylation, of the antigen in the different tissues of the reproductive tract.

Another possibility is that the antibodies detected different forms of the antigen. We reported earlier that the antibodies for the 110 and 74 kDa polypeptides reacted with their respective denatured subunit and with the native 180 kDa uterine protein under non-denaturing conditions (Kuivanen *et al.*, 1989). The antibody for the rat serum C3 protein reacted with serum C3 and the 180 kDa uterine protein under non-denaturing conditions (Kuivanen *et al.*, 1989), but did not react with the denatured 110 and 74 kDa polypeptide subunits (Kuivanen and DeSombre, unpublished observations). Previous studies with antibodies for human C3 demonstrated that antibodies for the denatured polypeptide subunits of C3 have sensitivities that are different from the sensitivity of an antibody raised to the native protein (Nilsson *et al.*, 1980; Nilsson and Nilsson, 1986). Antibodies to the denatured human C3 protein were more reactive with the bound substrate, while the antibody to the native human C3 protein was more reactive with the soluble antigen. It may be that the predominant form of the antigen in the endometrial and cervical epithelia is bound and more reactive with the antibodies to the denatured protein subunits, whereas the predominant form of the antigen in the vaginal epithelium is the soluble form of the antigen and more reactive with the antibody to native serum C3.

Another explanation is that there are multiple species of the antigen in the rat reproductive tract. From SDS-PAGE analyses, there is the suggestion that there may be multiple forms of the 180 kDa protein in the rat uterus. While serum C3 migrates as a single, 180 kDa protein on SDS-PAGE under non-denaturing conditions, the 180 kDa uterine protein migrates as a doublet (Lyttle *et al.*, 1987; Kuivanen *et al.*, 1989; Brown *et al.*, 1990). Western blot analysis revealed that both species of the doublet were immunoreactive with the three antibodies used in the present study (Kuivanen *et al.*, 1989). Analysis of the mRNA encoding the 180 kDa uterine protein also suggests that there is more than one species of protein. Sundstrom *et al.* (1989) used an LE-1 cDNA that mapped at the 3' end of C3 to probe northern blots of uterine RNA and RNA isolated from the luminal epithelium of oestrogen-treated immature rats and found a single 6.0 kb mRNA encoding C3 in the rat uterus. We, however, selected several cDNAs from a rat uterine cDNA gt11 expression library with a synthetic oligonucleotide based on the amino-terminal amino acid sequence of the 74 kDa polypeptide and used them as probes in northern blot analyses of uterine RNA from oestradiol-treated immature rats. These analyses revealed hybridization with multiple mRNA species that ranged in size, including 6.0 kb (data not shown).

The role of C3, or a C3-related protein, in the rat reproductive tract is unknown. It may provide protective immunity against infections of the mucosa of the reproductive tract, as has been described for immunoglobulins (Parr and Parr, 1985a, 1989). Experiments in rats have demonstrated an inhibition of bacterial growth after incubation of *Escherichia coli* in uteri of oestrous rats, but not in uteri of pseudopregnant rats (Wira and Merritt, 1977). In mice, the vagina is the source of most bacteria found in the uterus after mating (Parr and Parr, 1985b). Proteins related to C3 may therefore play important physiological roles in uterine–vaginal infections. In the endometriotic uterus, large amounts of C3 have been found (Badaway *et al.*, 1984; Hahn *et al.*, 1986; Isaacson *et al.*, 1990, 1991), suggesting that C3 or C3-related protein could be associated with the aberrant

proliferation of endometrial tissue. Complement-dependent sperm-immobilizing antibodies have been detected in the human cervical mucosa, suggesting that C3 or a C3-related protein may facilitate sperm mobility or survival in the cervix (Schumacher, 1980).

In summary, the results of the present study showed that (i) the 180 kDa uterine protein, of which the 110 kDa and 74 kDa polypeptide are subunits, was localized in the luminal and glandular epithelia of the endometrium and in the cervical and vaginal epithelia; (ii) in all of these epithelia, the 180 kDa uterine protein was regulated by oestradiol; (iii) C3 was present not only in the endometrial epithelium, but also in the cervical and vaginal epithelia, and was regulated by oestrogen in all three epithelia; (iv) the concentration of the proteins in these epithelia fluctuated during the oestrous cycle, being high during pro-oestrus and oestrus and undetectable during metoestrus and dioestrus consistent with their oestrogen regulation, and (v) the different immunoreactivities of the antibodies in the different tissues of the rat genital tract suggest that there may be multiple forms of the antigen in the different epithelia of the female rat reproductive tract. We are currently studying whether these forms originate as a result of transudation or local synthesis and whether they represent different processing or biologically functional forms of the protein.

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