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Involvement of Fas in regression of vaginal epithelia after ovariectomy and during an estrous cycle

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Fas, also called APO-1, belongs to the tumor necrosis factor/nerve growth factor receptor family and transmits an apoptotic signal within the cell by binding to the Fas ligand. Fas has been implicated in the activation-induced suicide of T cells and cytotoxic T cell activity in the immune system. Non-immune cells such as those in liver, lung and ovary also express Fas, but its role in these cells remains unclear. Ovariectomy has been used to study homeostasis of female reproductive organs, which is regulated by sex hormones. Here we analyzed Fas function in the ovariectomy-induced regression of mouse vaginal epithelial cells. Fas expression was detected in vagina and was elevated after ovariectomy. Fas-deficient *lpr* and *lpr^{cg}* mice did not exhibit ovariectomy-induced regression of vaginal epithelia, whereas uterine regression induced by ovariectomy was not affected in these mice. The vaginas of *lpr* and *lpr^{cg}* mice were in a persistent estrous stage with cornification of vaginal epithelia, as judged from the cell types in the vaginal fluid. Thus, Fas appears to be involved directly in the regression of vaginal epithelia induced by ovariectomy and during the estrous cycle, suggesting that the physiological role of this receptor extends beyond that exerted on immune cells. This is the first evidence of a role for Fas in inducing physiological apoptosis in non-immune cells.

Keywords: apoptosis/Fas/*lpr*/vagina

Introduction

Programed cell death is a physiological process indispensable for normal development and homeostatic maintenance of multicellular organisms, most of which proceed in an apoptotic fashion. Apoptotic cell death is characterized by morphological alterations including membrane blebbing, chromatin condensation, nuclear fragmentation and generation of apoptotic bodies. Several genes involved in apoptosis have been described, some functioning to drive the death machinery and others acting against apoptotic cell death.

Two cell surface cytokine receptors, Fas/APO-1 (Itoh *et al.*, 1991; Oehm *et al.*, 1992; Watanabe-Fukunaga *et al.*, 1992b) and the receptor for tumor necrosis factor (TNF) (Tartaglia and Goeddel, 1992), both of which belong to the TNF/nerve growth factor (NGF) receptor family, transmit an apoptotic signal within the cell by binding to the natural ligand or to specific agonist antibodies (Trauth *et al.*, 1989; Suda *et al.*, 1993). Mice with loss-of-function mutations of Fas (*lpr* and *lpr^{cg}*) (Watanabe-Fukunaga *et al.*, 1992a,b), and Fas ligand-deficient mice (*gld*) (Takahashi *et al.*, 1994) exhibit a lymphoproliferative autoimmune disorder, and the Fas/FasL system has been implicated in activation-induced suicide of T cells (Russell *et al.*, 1993; Singer and Abbas, 1994; Brunner *et al.*, 1995) and cytotoxic T cell action (Kagi *et al.*, 1994; Lowin *et al.*, 1994) in the immune system. Non-immune cells such as those in liver, lung and ovary also express Fas (Watanabe-Fukunaga *et al.*, 1992b), but its role in these cells remains unclear. Whereas the main activity of Fas is to transmit the apoptotic signal, the TNF receptor is able to transmit both proliferative and apoptotic signals.

Cell proliferation and regression of female reproductive organs are regulated by sex hormones (Takasugi, 1976), providing a useful system to study cell death. It remains unclear how female reproductive organs such as the vagina and uterus regress upon withdrawal of sex hormones. Here, we show that Fas is expressed in vaginal cells and required for vaginal regression induced by ovariectomy and also for physiological vaginal regression during an estrous cycle.

Results

Involvement of Fas in vaginal but not uterine regression

To test the involvement of Fas in the regression of vaginal epithelial cells, two lines of Fas-deficient mice, *lpr* and *lpr^{cg}*, were analyzed, in which the Fas gene is interrupted by an early transposable element (Adachi *et al.*, 1993) and carries a point mutation in the death domain (Watanabe-Fukunaga *et al.*, 1992a), respectively. Ovariectomy of normal mice at estrous stage resulted in decreased organ weight of vagina and uterus (Figure 1). Organ weight of uterus from 3-month-old *lpr* and *lpr^{cg}* mice decreased after ovariectomy to the same extent as in normal mice, whereas the weight of the vagina remained unchanged (Figure 1), suggesting the involvement of Fas in vaginal but not uterine regression. Consistent with this observation, Fas expression was detected in vagina of normal mice and was elevated after ovariectomy but was not detected in uterus before or after ovariectomy (Figure 2a). Interestingly, bcl-2 expression, which is able to block Fas-induced

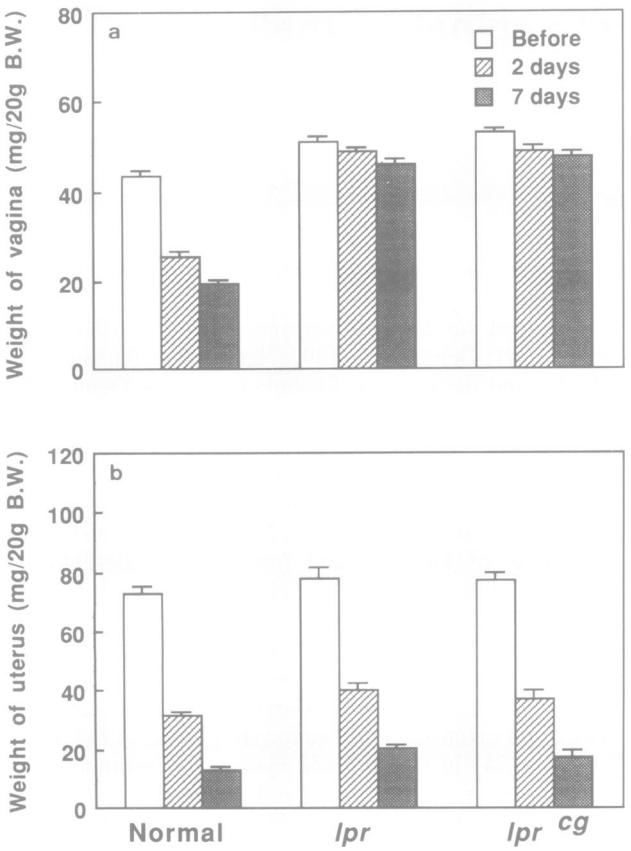

Fig. 1. Change in weight of vagina and uterus after ovariectomy. Normal, *lpr* and *lpr*^{cg} mice were ovariectomized at an estrous stage. The organ weight of vagina (**a**) and uterus (**b**) was measured before and 2 and 7 days after ovariectomy, and expressed as mg per 20 g body weight (mean with SE). Seven normal, seven *lpr* and five *lpr*^{cg} mice were studied at each experimental point. Results with normal CBA mice were virtually identical to those with C57BL/Tw mice.

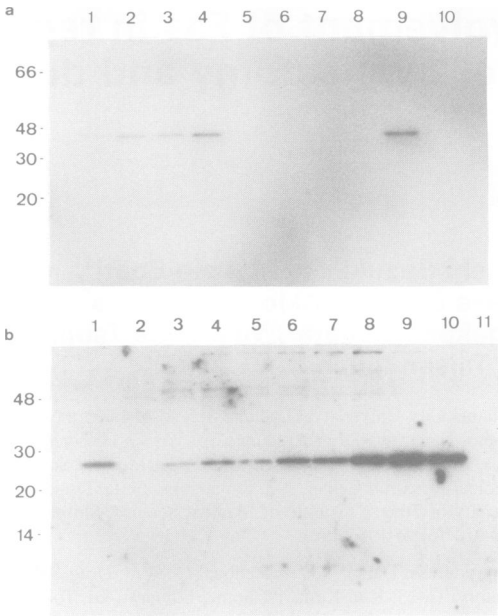

Fig. 2. Fas and bcl-2 expression in vagina and uterus before and after ovariectomy. Normal 8-week-old C57BL mice were ovariectomized at estrous stage, and Fas (45 kDa) (**a**) and bcl-2 (26 kDa) (**b**) expression in vagina and uterus was examined by immunoprecipitation and Western blotting, respectively as described in Materials and methods. (a) Lanes 1–4, vagina; lanes 5–8, uterus; lane 9, thymus known to express Fas; lane 10, brain known not to express Fas. Lanes 1 and 5: before ovariectomy; lanes 2 and 6: 2 days after ovariectomy; lanes 3 and 7, 7 days after ovariectomy; lanes 4 and 8, 14 days after ovariecomy. (b) Lanes 1–8, the same as those in (a); lane 9, spleen known to express bcl-2; lane 10, brain known to express bcl-2; lane 11, skeletal muscle known not to express bcl-2. Size of molecular markers is given in kDa.

apoptosis (Itoh *et al.*, 1993), was down-regulated in vagina of normal mice 2 days after ovariectomy and restored to initial levels in 2 weeks (Figure 2b). In the uterus, the bcl-2 expression continued to increase without any down-regulation for at least 2 weeks after ovariectomy (Figure 2b).

Histological analysis of the vagina before and after ovariectomy revealed >10 epithelial cell layers with keratinized superficial layers in the vagina of normal mice at estrous stage, whereas ovariectomized normal mice at an estrous stage showed complete loss of epithelial layers of the vagina after 7 days, without any significant morphological change in stromal layers (Figure 3). In contrast, vagina of *lpr* and *lpr*^{cg} mice consistently revealed >10 layers of cells with superficial keratinized layers even 7 days after ovariectomy (Figure 3). These results suggest that Fas is directly involved in ovariectomy-induced loss of vaginal epithelia.

Association of apoptosis in vaginal regression

To determine whether ovariectomy-induced loss of vaginal epithelial cells is accompanied by apoptotic cell death, we analyzed nuclear DNA extracted from vagina of normal, *lpr* and *lpr*^{cg} mice. Oligonucleosomal DNA fragmentation, which frequently is associated with apoptosis (Wyllie *et al.*, 1980) was observed in DNA from vagina of normal mice 2 days after ovariectomy, but not in DNA from vagina of ovariectomized *lpr* and *lpr*^{cg} mice (Figure 4), suggesting that apoptotic cell death plays an important role in the regression of vaginal epithelia. No DNA fragmentation was detected in uterus of normal or *lpr* mice after ovariectomy (data not shown).

Involvement of Fas in regression of vaginal epithelia during a normal estrous cycle

Direct involvement of Fas in the ovariectomy-induced regression of vaginal epithelia raised the question of whether Fas is also involved in the regression of vaginal epithelia that occurs during a normal estrous cycle. Light microscopy analysis of vaginal smears obtained periodically from normal and *lpr* mice showed typical changes in cell types during an estrous cycle of normal mice, confirming the previous observation (David, 1980), whereas the vaginal smear of *lpr* mouse consistently revealed mainly keratinocytes typical of the estrous stage (Figure 5). This suggests that the lack of functional Fas leads to failure of vaginal epithelia to regress during an estrous cycle.

Discussion

We have shown, using Fas-deficient mice, that Fas is directly involved in vaginal regression induced by ovariectomy. Consistent with this demonstration was the detection of oligonucleosomal DNA fragmentation during

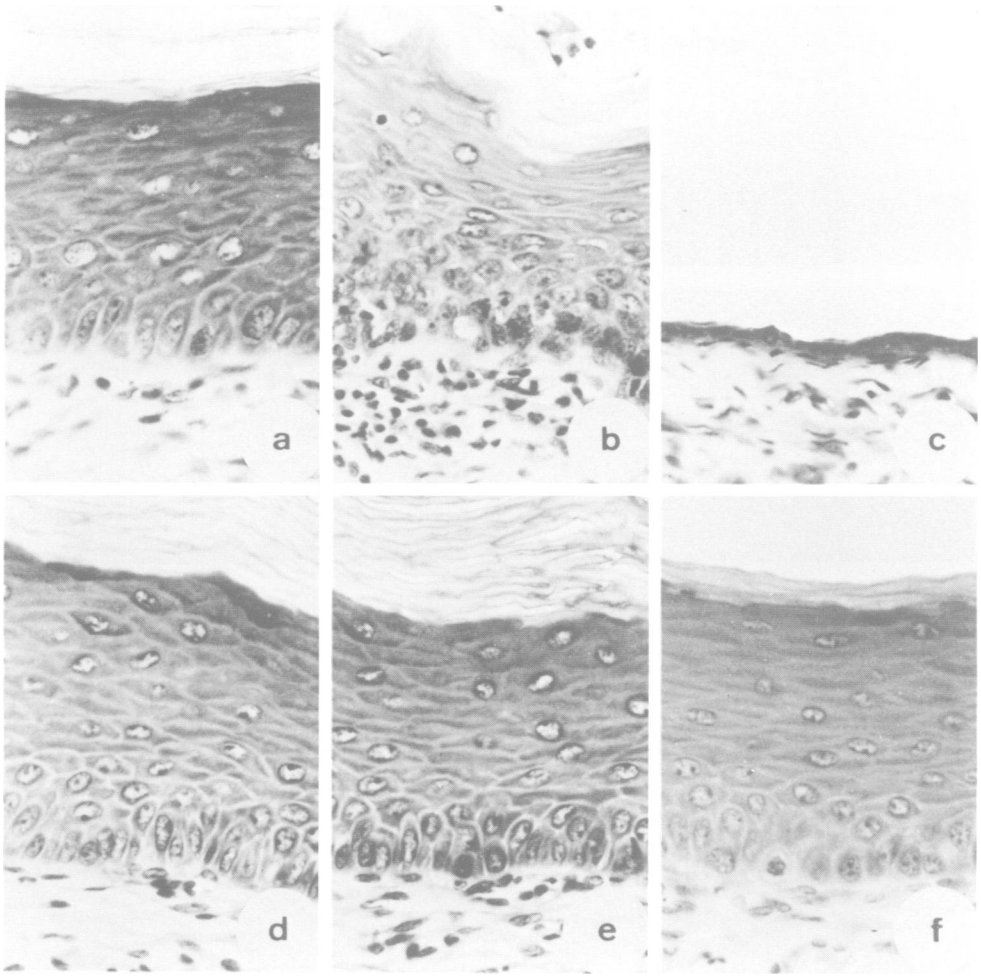

Fig. 3. Histological analysis of vagina after ovariectomy. Micrographs of cross-sections of vagina from normal (**a**, **b** and **c**) and *lpr* mice (**d**, **e** and **f**) were taken before (a and d) and at 2 days (b and e) and 7 days (c and f) after ovariectomy. Original magnification, 300×.

vaginal regression, suggesting that apoptosis plays a major role in vaginal regression. Since no evidence of vaginal regression was found in *lpr* mice at least 14 days after ovariectomy, as assessed by histological examination, the process of regression appears to be completely blocked but not delayed by the absence of Fas. The down-regulation of bcl-2 expression in vagina after ovariectomy might represent a mechanism to facilitate Fas-mediated apoptosis. Although bcl-2 expression following the rapid down-regulation after ovariectomy was restored in vagina, infiltrating leukocytes or stromal cells of the vagina might be the source of this expression because epithelial layers are lost by cell death in 7 days after ovariectomy. Fas transduces the death signal by binding to Fas ligand. Since an infiltration of leukocytes was observed 2 days after ovariectomy in the vagina of a normal mouse, Fas ligand expressed on the cell surface of the infiltrating leukocytes may be responsible for apoptosis of vaginal epithelial cells. It would, therefore, be of interest to examine vaginal regression in ovariectomized *gld* mice. Further studies are needed to determine how Fas is activated in vaginal epithelial cells.

Ovariectomy still induced a decrease of organ weight in the uterus of *lpr* and *lpr*^{cg} mice, suggesting that uterine regression after ovariectomy is caused by a mechanism

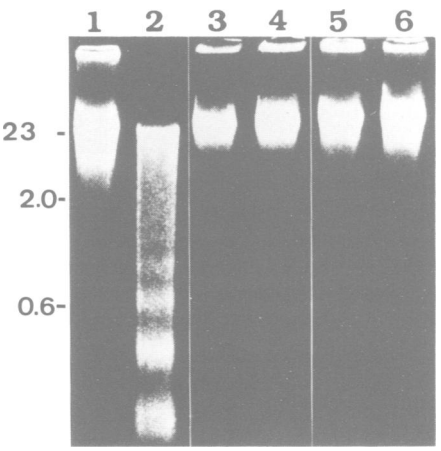

Fig. 4. Nuclear DNA fragmentation in vagina after ovariectomy. DNA was extracted from vagina of normal (lanes 1 and 2), *lpr* mice (lanes 3 and 4) and *lpr*^{cg} mice (lanes 5 and 6) before (lanes 1, 3 and 5) and 2 days after ovariectomy (lanes 2, 4 and 6) and separated on a 2% agarose gel. DNA was visualized by staining with ethidium bromide. The size of marker DNA is in kilobases.

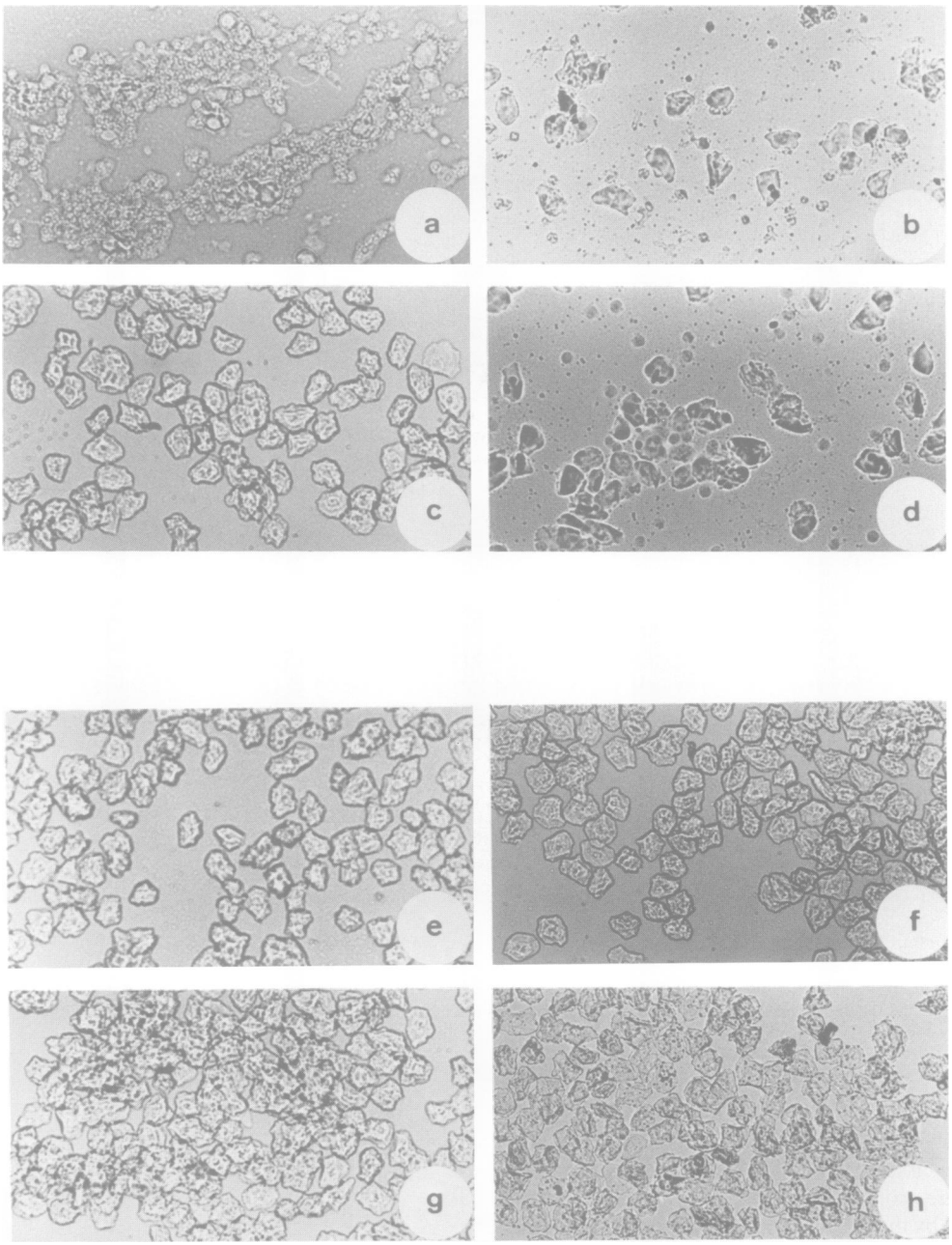

Fig. 5. Microscopic examination of vaginal smears from normal and *lpr* mice. A vaginal smear was collected with cotton buds on four successive days from a single normal (a–d) and *lpr* mouse (e–h), and smears were prepared on glass slides for microscopic observation. (**a**) Diestrous stage with lymphocytes remaining in the vaginal fluid; (**b**) proestrous stage in which vagina consists of ~10 epithelial layers and the upper layer begins to be keratinized; therefore, vaginal fluid contained live nucleated epithelial cells; (**c**) estrous stage in which vaginal epithelial cells at upper layers are keratinized, and cells in the vaginal fluid were mostly keratinocytes; and (**d**) metestrous stage in which the number of epithelial layers decreases to four or five, and keratinocytes and lymphocytes were observed in vaginal smears. (**e–h**) All vaginal smears from the *lpr* mouse revealed mainly keratinocytes throughout the period examined, indicating that the vagina persists in an estrous stage.

that does not involve Fas, consistent with the absence of Fas expression in the uterus of a normal mouse before and after ovariectomy. Because extensive shrinkage of epithelial and stromal cells occurs during uterine regression (not shown), and DNA fragmentation was not observed in uterus after ovariectomy, cell death might not account for most of the uterine regression.

Our data show that the lack of functional Fas abrogates the normal changes in vaginal epithelia during an estrous cycle. Thus, the naturally occurring regression of vaginal epithelial cells during estrous cycles is mediated by Fas. This is the first observation that Fas plays a dominant role in a physiological apoptotic process in non-immune cells, raising the possibility that Fas plays an important homeostatic role in a variety of tissues. More detailed studies are required on Fas expression in various organs, including those previously thought to be negative for Fas expression.

Materials and methods

Mice

Female C57BL/Tw, C3H/HeJ-*lpr*/*lpr* and CBA/KlJms- lpr^{cg}/lpr^{cg} mice were used. C3H/HeJ-*lpr*/*lpr* mice were obtained from the Jackson

Laboratory (Bar Harbor, ME) and bred in the Animal Research Center at the Institute of Medical Science, University of Tokyo. CBA/KlJms-*lpr*^{cg}/*lpr*^{cg} mice have been established and maintained in the same center (Matsuzawa *et al.*, 1990). Mice were kept in 12 h light–12 h dark at 23–25°C and given standard laboratory chow (CE-2, CLEA, Tokyo) and tap water *ad libitum*. Vaginal smears were examined in a 3 week period from 2 months of age. C57BL mice at estrous stage, as judged by vaginal smears, and 3-month-old *lpr* mice were ovariectomized.

Immunoprecipitation

Immunoprecipitation of Fas antigen was carried out as described (Ogasawara *et al.*, 1993). Briefly, vaginal cell surface proteins were labeled with biotin, and lysed in buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA and 1% NP-40). One mg of whole protein was precleared with normal hamster IgG (8 mg) and immunoprecipitated with 1 μg of the Jo2 anti-Fas antibody. The immunoprecipitates were separated on 10% SDS–polyacrylamide gels, transferred onto nitrocellulose membranes and incubated with avidin-conjugated alkaline phosphatase (Vector Labs, Burlingame, CA). Filters were washed with 0.5% Tween–phosphate-buffered saline (PBS) and then incubated at room temperature for 5 min in a reaction mixture containing 5-bromo-4-chloro-3-indolyl-phosphate (Sigma) and nitroblue tetrazolium (Wako) to develop colored product on the filters.

Western blotting

Mouse bcl-2 protein was detected by Western blotting using anti-glutathione S-transferase mouse bcl-2β polyclonal rabbit antibody (affinity-purified) as described (Kamada *et al.*, 1995). Tissues were lysed with 8 M urea/1% SDS/2.5% dithiothreitol, and NP-40 was added to 10%. Four μg of protein was loaded on 12.5% SDS–polyacrylamide gels.

DNA analysis

Tissues were homogenized and lysed in buffer containing 100 mM NaCl, 25 mM EDTA, 100 mM Tris–HCl (pH 8.0), 0.5% SDS and 0.3 mg/ml proteinase K at 50°C for 14 h. Samples were extracted three times with phenol–chloroform–isoamyl alcohol (PCIA; 25:24:1) and once with chloroform. After adding an equal volume of isopropanol to the aqueous phase, DNA was precipitated and dissolved in 10 mM Tris–HCl, 50 mM EDTA (pH 8.0) and treated with 50 μg of RNaseA for 1 h at 37°C. After PCIA extraction, DNA was precipitated as described above. Ten μg of DNA was separated on a 2% agarose gel in TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA).

Histology

Vaginas were fixed in Bouin's solution, embedded in paraffin and serially sectioned at 8 μm. Sections were stained with Delafield's hematoxylin and eosin.

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