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Regulation of the estrous cycle by neutrophil infiltration into the vagina

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ABSTRACT

During metestrus of the estrous cycle, a number of neutrophils infiltrate into the vaginal vault, presumably due to a neutrophil-specific chemokine, MIP-2, in mice. The physiological role of the infiltrating neutrophils, however, remains largely obscure. In this study we examined the effects of neutrophil depletion on the estrous cycle and steroid hormone levels. When mice were treated with an anti-Gr-1 mAb, they became neutropenic, as assessed as to the number of neutrophils in the peripheral blood. The estrous cycle of such mice was specifically blocked at diestrus irrespective of the phase at which the anti-Gr-1 mAb was administered. The blockade was reversible, because restoration of neutrophils to a normal level caused a restart of the cycle. Immunohistochemical analyses revealed that neutrophils were present mainly on the luminal surface and in the lumen at metestrus and to a lesser extent at diestrus but scarcely in the uterine cervix at any phase, and that the anti-Gr-1 mAb depleted neutrophils but not eosinophils in the vagina. The treatment with the anti-Gr-1 mAb significantly affected the serum 17β-estradiol and progesterone levels at diestrus after the estrous cycle was blocked. Together, these results suggest that neutrophil infiltration into the vagina is critical in maintaining the estrous cycle through control of steroid hormone levels.

Introduction

Neutrophils are situated as the first line of host defense against bacterial infection by virtue of phagocytosis and production of reactive oxygen species. For neutrophils to perform these functions, it is very critical that CXC chemokines responsible for neutrophil infiltration are produced at appropriate times and places. Bacterial infection of the lungs, for instance, causes rapid production of such CXC chemokines in them, leading to acute accumulation of neutrophils at the sites of infection [1,2].

It is well known that neutrophils infiltrate into the vaginal vault at metestrus, as seen on vaginal smearing for determination of the phases of the estrous cycle, namely proestrus, estrus, metestrus, and diestrus [3,4]. The neutrophil infiltration is mediated by macrophage inflammatory protein 2 (MIP-2), one of the CXC chemokines specific to neutrophils in mouse [5]. Recent research has led to the identification of several chemokines in the rat ovary that are hormonally regulated, among which GRO, one of the chemokines specific to neutrophils in rat, is expressed markedly in the preovulatory rat ovary [6], although it is not known whether or not GRO is responsible for neutrophil infiltration into the vagina in rat.

Although infiltrating neutrophils are expected to keep the vaginal vault pathogen-free, there have been many reports that infiltrating neutrophils do not play a major role in clearance of *C. albicans* in the vaginal vault [7,8]. Consequently, the physiological role of neutrophil infiltration remains to be elucidated. In this study, we examined the effects of neutrophil depletion on the estrous cycle and serum steroid hormone levels.

Materials and methods

Mice. Specific pathogen-free female ICR mice (5–7 wks old) were purchased from Sankyo Lab Service (Tokyo, Japan). The mice were then maintained under a 12:12 light-dark cycle (lights on from 7 am to 7 pm), and vaginal smears were prepared daily at 10 am for 3 estrous cycles, usually 12–15 days. In this study, we used mice that had completed at least 2 estrous cycles. The project was approved by the Animal Experiment Committee of Toho University.

Determination of each phase of the estrous cycle. Each phase of the estrous cycle was determined by analysis of vaginal smears. Methanol-fixed smears were stained with a Diff–Quik staining solution according to the manufacturer's instructions. Each phase of the estrous cycle was defined as follows: proestrus (100% intact epithelial cells), estrus (100% cornified epithelial cells), metestrus (~50% cornified epithelial cells or exfoliated epithelial cells and 50% leukocytes), and diestrus (cell debris, some cornified epithelial cells or leukocytes). The proestrus and estrus phases were also assessed as to the appearance of the vagina [9,10].

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Neutrophil depletion. To deplete neutrophils, 200 μg/ 0.2 ml of a rat anti-neutrophil monoclonal antibody (anti-Gr-1 mAb), which was prepared from supernatants of RB6-8C5 cells (the cells were provided by Dr. Sendo of Yamagata University), was administered intraperitoneally 18 h before examining vaginal smears and neutropenia was checked with Giemsa's method. As a control, an equal dose of an anti-HLA mAb, which was prepared from supernatants of SFR8-B6 cells (the cells were obtained from ATCC), was administered intraperitoneally at the same time.

Immunohistochemistry. After organs had been cleared of fat, they were cut into pieces and fixed in cold Zamboni's fixative for 6 h, and then delipidated with methanol and chloroform (1:1) at 4 °C overnight. The tissues were then dehydrated using a graded dehydration series of ethanol. They were made transparent with xylene followed by immersion in Pathoprep® 568 (Wako, Osaka, Japan). The tissues embedded in paraffin were cut into 2-μm thick sections. The sections were placed on silane-coated Superfrost microslide glasses® (Matsunami, Tokyo, Japan) and then air-dried at 37 °C overnight. They were then deparaffinized and rehydrated. After washing, they were immersed in freshly prepared 0.3% H_2O_2 in PBS containing 10% methanol for 15 min. To retrieve Ags, the sections were incubated with an L.A.B. solution (Polysciences Inc., Warrington, PA) for 15 min at rt. During the subsequent steps, the tissue sections were kept under humid conditions. After washing the sections, the section was incubated with a 3% BSA solution for 30 min at rt. to reduce the background staining. Then a rat anti-Gr-1 mAb or rabbit anti-myeloperoxidase (MPO) antibody (Ab) (Lab Vision, Fremont, CA) was applied to the slides at a dilution of 2 μg/ml or 1:200 dilution, respectively, followed by incubation at 4 °C overnight. After washing, the sections were each treated with 5 μg/ml biotinylated secondary antibodies (American Qualex, San Clement, CA) for 60 min at rt. And then, VECTASTAIN elite ABC Kit (Vector Laboratories, Inc., Burlingame, CA) and Diaminobenzidine (DAB) Substrate Kit (Vector) was used according to the manufacturer's instructions.

When necessary, Congo red stain was used to confirm the presence of eosinophils. Briefly, the sections were stained with 0.5% Congo red in 80% ethanol for 15 min and then differentiated with an alcoholic potassium hydroxide solution quickly. In this case, a Vector Blue kit (Vector) and ABC-AP Kit (Vector) were used instead of the DAB and ABC Kit described above.

As a control, some of the sections were incubated with either normal rabbit IgG or normal rat IgG.

Hormone measurement. The serum 17β-estradiol and progesterone levels were determined by means of an Estradiol EIA kit or a Progesterone EIA kit (Cayman Chemical, Ann Arbor. MI). The detection limits for estradiol and progesterone were 8 pg/ml and 10 pg/ml, respectively.

Statistics. Differences between experimental groups were analyzed by means of one-way factorial analysis of variance (one factor ANOVA) and the post-hoc test (Scheffe's F) using Statcel (OMS-publishing, Saitama, Japan).

Results

The estrous cycle of neutrophil-depleted mice

Each estrous phase was determined by cytological analysis of vaginal smears and continues for one day except for metestrus that sometimes continues for two days. In such cases, metestrus is subdivided into metestrus-1 and -2. Among the four phases, metestrus is defined as the phase at which a number of neutrophils infiltrate into the vaginal vault.

We then examined the estrous cycle in neutrophil-depleted mice. To deplete neutrophils, an anti-Gr-1 mAb was administered at days 0 and 3, as indicated by open circles (Fig. 1). Under these conditions, mice remained neutropenic till day 5 or day 6 (data not shown). A control mAb did not cause neutropenia.

When the anti-Gr-1 mAb was administered at proestrus or estrus, the estrous cycle continued up to diestrus, and thereafter was blocked at the diestrus (Fig. 1A and B). At day 7, the phase changed to proestrus, suggesting that restoration of the percentage of neutrophils led to a restart of the estrous cycle.

When the anti-Gr-1 mAb was administered at metestrus, however, the estrous cycle was not blocked at the nearest diestrus. In addition, when the anti-Gr-1 mAb was administered at diestrus, the estrous cycle was not blocked at that very diestrus instantaneously. In these two cases, the estrous cycle was completed once, and then proceeded to the next diestrus and thereafter was blocked at the diestrus (Fig. 1C and D). Although the data are not shown, the phase changed to proestrus at day 8, suggesting again that restoration of the percentage of neutrophils led to a restart of the estrous cycle. When the anti-HLA mAb was administered as a control, the estrous cycle was not affected (Fig. 1E). It should be noted that, in neutrophil-depleted mice, each phase of the estrous cycle is determined by the changes in epithelial cells in vaginal smears and the appearance of the vagina.

Although anti-MIP-2 antibodies tended to suppress neutrophil infiltration into the vagina, the estrous cycle was hardly affected, presumably due to incomplete suppression of neutrophil infiltration (data not shown), being in agreement with the previous paper [5].

Immunohistochemical analysis of vaginal sections of mice treated with an anti-Gr-1 mAb

Although neutrophil depletion was confirmed with smears of tail blood and vaginal smears of mice treated with an anti-Gr-1 mAb, the possibility remains that other leukocytes such as eosinophils are also depleted in the vagina, because eosinophils have been found to be Gr-1^{low} on flow cytometric analysis [11,12]. We therefore examined vaginal sections of mice at metestrus by staining with the anti-Gr-1 mAb, anti-MPO pAb, and Congo red in Fig. 2. Staining with Congo red is known to be specific for eosinophils [13], whereas neutrophils but not eosinophils were stained with the anti-Gr-1 mAb on immunohistochemical analyses of tail blood and a leukocyte-rich population isolated from the uterus (data not shown).

The cells stained with the anti-Gr-1 mAb, neutrophils, were localized to the luminal surface and lumen of the vagina (Fig. 2A and E). On the other hand, there was no staining with the anti-Gr-1 mAb in the sections of mice treated with the anti-Gr-1 mAb (Fig. 2C and D), suggesting that no neutrophils remain in the vagina of mice treated with the anti-Gr-1 mAb. To further confirm the absence of neutrophils in the vagina, the sections were stained with anti-MPO pAb. Contrary to our expectation, the anti-MPO pAb detected not only neutrophils but also other cells, eosinophils, in the sections of control mice (Fig. 2B), and the anti-MPO pAb detected eosinophils in the sections of mice treated with the anti-Gr-1 mAb (Fig. 2D), presumably because the pAb crossreacts with peroxidase in eosinophils. Eosinophils were stained with Congo red, and were found to be localized to the vaginal and uterine stroma in the sections of control mice, whereas the anti-Gr-1 mAb stained neutrophils but not eosinophils (Fig. 2E). In Fig. 2E, neutrophils were stained blue by the anti-Gr-1 mAb. Therefore, the anti-Gr-1 mAb depleted neutrophils but not eosinophils in the vagina.

Distribution of neutrophils and eosinophils in the vaginal opening and uterine cervix of mice at various phases of the estrous cycle

We then determined the distribution of neutrophils and eosinophils in the vaginal opening at various phases by means of an anti-Gr-1 mAb and an anti-MPO pAb.

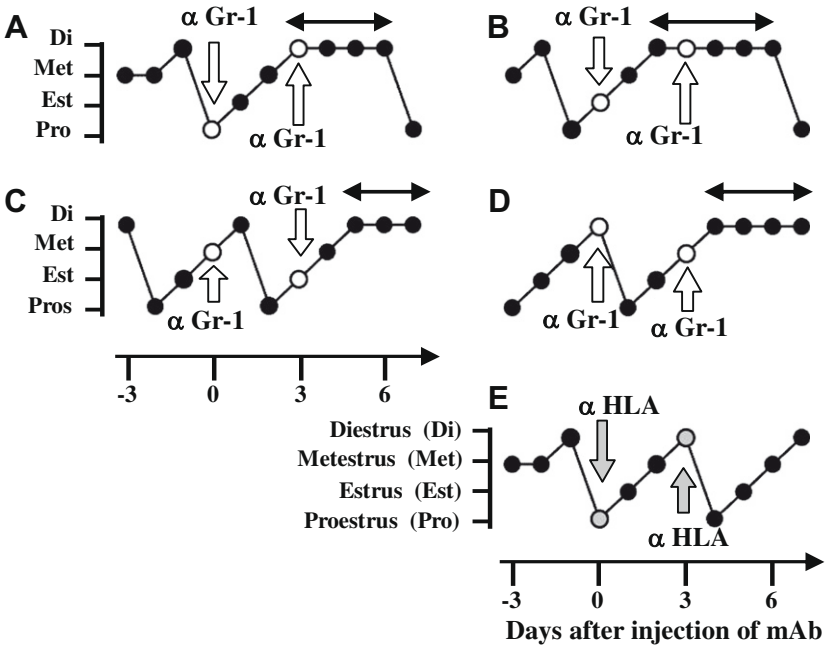

Fig. 1. Changes of the estrous cycle of anti-Gr-1 mAb-treated mice. Mice were treated with anti-Gr-1 mAb (A–D) or control mAb (E) at proestrus (A), estrus (B), metestrus (C) or diestrus (D) on day 0. The mice were also treated with anti Gr-1 mAb or control mAb on day 3. The estrous cycle was determined in each mouse every day. We repeated the experiments over 10 times. The representative result was shown.

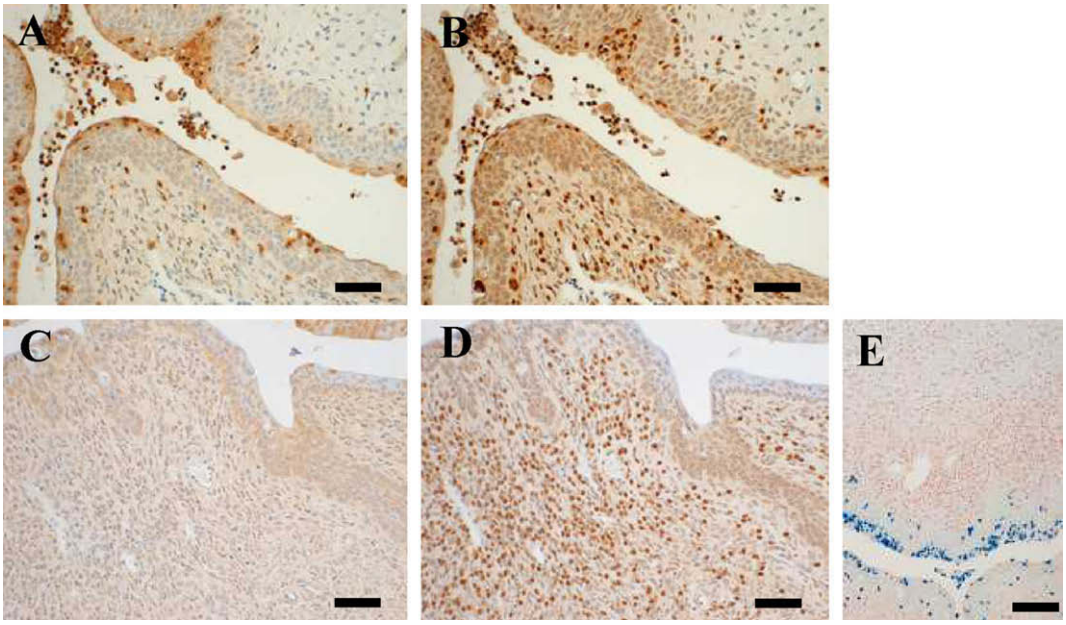

Fig. 2. Immunohistochemical analysis of vaginal sections of mice at metestrus. Mice at estrus were treated with either an anti-Gr-1 mAb (C, D) or an anti-HLA mAb (A, B, and E). On the next day, vaginal sections were prepared and stained with an anti-Gr-1 mAb (A, C, and E), an anti-MPO pAb (B, D), or Congo red (E), as described under Materials and Methods. In panel E, neutrophil signals are blue, whereas eosinophil signals are red. Scale bar, 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

At the border of the vaginal opening, neutrophils were detected mainly on the luminal surface and in the lumen at metestrus (Fig. 3B), and to a lesser extent at diestrus (Fig. 3D), whereas eosinophils were detected not on the luminal surface and in the lumen but in the stroma of mice at metestrus (Fig. 3A vs. B) and diestrus (Fig. 3C vs. D). The localization of eosinophils was further confirmed by Congo red staining (data not shown). At proestrus and estrus, there were few neutrophils in the vaginal opening (Fig. 3E and F). Although the data are not shown, a few neutrophils and many eosinophils were detected in the uterine cervix at any phase.

Changes in the serum 17β-estradiol and progesterone levels in neutrophil-depleted mice

The estrous cycle is under the control of the hormone system. Because neutrophil depletion caused blockade of the estrous cycle, we

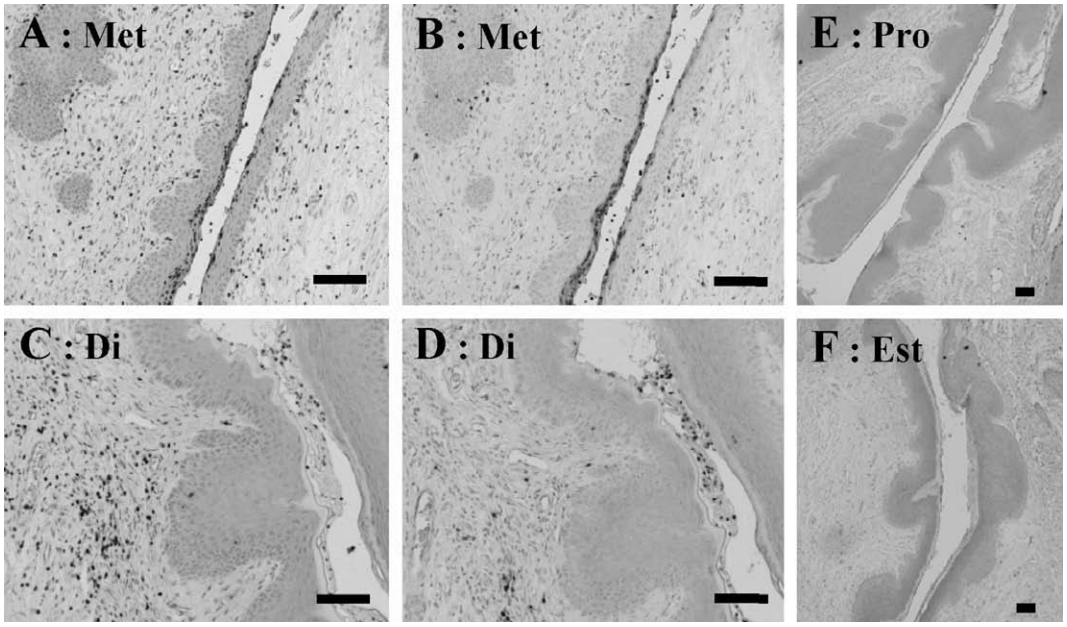

Fig. 3. Distribution of neutrophils and eosinophils in the vaginal openings of mice at various phases of the estrous cycle. Sections of the vaginal opening of mice at various phases of the estrous cycle were prepared and stained with either an anti-MPO pAb (A, C) or an anti-Gr-1 mAb (B, D, E, F) as described under Materials and methods. Scale bar, 100 μm.

then examined the serum 17β-estradiol (E2) and progesterone (P4) levels in neutrophil-depleted and control mice. The E2 level peaked at estrus in control mice, whereas, after blockade of the estrous cycle, the E2 level remained at the level at metestrus and then gradually returned to the level at diestrus (Fig. 4A). In contrast, the P4 level peaked at metestrus-1 in control mice, whereas, after blockade of the estrous cycle, the P4 level remained at the level at metestrus-1 and then gradually returned to the level at diestrus (Fig. 4B). The serum E2 and P4 levels were not altered, however, in neutrophil-depleted mice in which the estrous cycle had not been blocked yet (data not shown). Additionally, there were histological changes in the ovary during the estrous cycle of control mice but not mice in which the estrous cycle was blocked (data not shown).

Discussion

Neutrophil depletion led to blockade of the estrous cycle at diestrus, which was presumably caused by a lack of neutrophil infiltration into the vagina and vaginal vault at metestrus. Further, neutrophil depletion affected the serum levels of E2 and P4 at diestrus, and the levels gradually returned to the normal levels at diestrus during blockade of the estrous cycle even though the mice remained neutropenic.

In this study, we used an anti-Gr-1 mAb to deplete neutrophils. Although it has been reported that this mAb binds to and depletes neutrophils and eosinophils but not lymphocytes and macro-

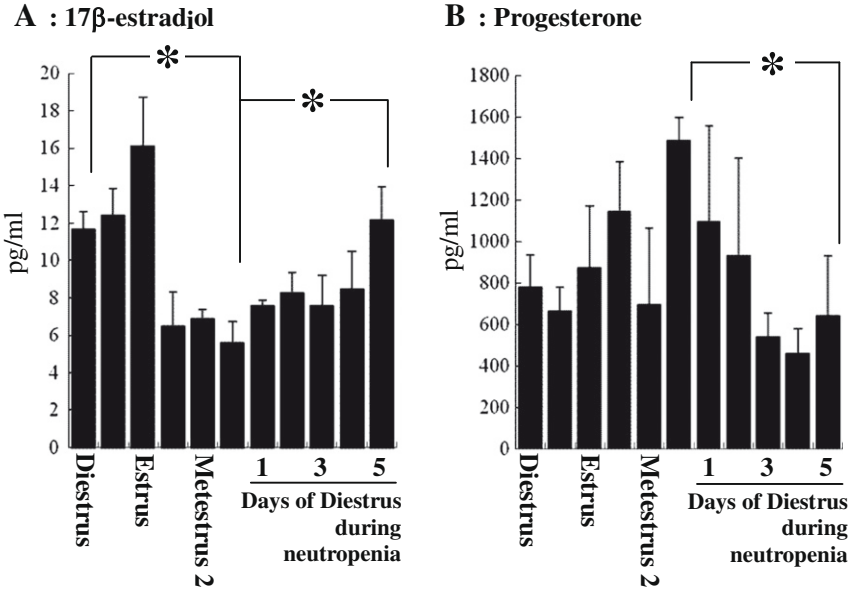

Fig. 4. Changes in the serum E2 and P4 levels in neutrophil-depleted mice. Sera were collected from control or anti-Gr-1 mAb-treated mice at various phases of the estrous cycle, followed by determination of the E2 (A) and P4 (B) levels by specific ELISAs. The numbers after Diestrus indicate the time in days after blockade of the estrous cycle. The data are expressed as the means ± standard errors for 3–5 mice. $^{*}p < 0.05$.

phages [14,15], we found that neutrophils but not eosinophils were depleted from the vagina and uterine cervix of anti-Gr-1 mAb-treated mice. It should be noted that we used uninfected mice, whereas others used infected [14] or immunized mice [15], although the precise reason for this discrepancy is not known at present.

It has been reported that infiltration of eosinophils into rat, mouse, and human uteri was coincident with the estrous cycle [16,17]. Further, treatment of ovariectomized mice with E2 resulted in an increase in eosinophils [16,18], and eotaxin was identified as the E2-induced chemokine responsible for eosinophil homing to the uterine stroma [19]. However, it has been reported that eosinophils do not play a role in regulating proper cyclicity in the adult uterus [19]. Given these reports, it is unlikely that eosinophils are also involved in blockade of the estrous cycle of anti-Gr-1 mAb-treated mice.

The timing of administration of the anti-Gr-1 mAb appears to determine when the estrous cycle is blocked. The most likely scenario is as follows. At proestrus or estrus, there are no neutrophils in the vagina or uterus to deliver the signal for production of steroid hormones in the vagina or uterus. Therefore, if the anti-Gr-1 mAb is administered at proestrus or estrus, the estrous cycle continues up to diestrus, and thereafter is blocked at the diestrus. At metestrus or diestrus, on the other hand, neutrophils are transversing or have just transversed the vagina or uterus, and thus it is assumed that neutrophils are delivering or have just delivered the signal in the vagina or uterus at the metestrus or diestrus, respectively. Therefore, if the anti-Gr-1 mAb is administered at metestrus or diestrus, the estrous cycle is not blocked at the nearest diestrus or that very diestrus. Rather, the estrous cycle is completed once, then proceeds up to diestrus and thereafter is blocked at that diestrus. In support of this scenario, even when mice were treated with the anti-Gr-1 mAb at estrus, in some cases neutrophils were detected in vaginal smears of mice at the nearest metestrus, which suggests that neutrophils existed in the vagina or uterus at estrus when the anti-Gr-1 mAb was administered, and in such cases the estrous cycle was not blocked at the nearest diestrus. Indeed in some cases neutrophils were found on the wall of the uterine cervix at estrus. Together, these results suggest that the presence of neutrophils in the vagina or uterus between estrus and metestrus is required to maintain the regular estrous cycle.

The serum E2 and P4 levels as well as the morphological changes in epithelial cells in the vagina and vulva were affected when the estrous cycle was blocked. This was in good agreement with the finding that E2 and P4 affect the morphological changes in epithelial cells in the vagina and vulva [10]. Importantly the level of each hormone returned to the normal level afterwards even though the mice remained neutropenic, indicating the existence of another pathway that regulates the estrous cycle, which is independent of neutrophil infiltration.

There is a possibility that the blockade of the estrous cycle seen in this study was due to pseudopregnancy. The plasma P4 level in pseudopregnant mice rose significantly soon after the induction of pseudopregnancy and reached a plateau on days 4–5, while in pregnant mice, it gradually increased to a peak on days 15 and 16 [20]. But in this study, after blockade of the estrous cycle, the serum P4 level remained at the level at metestrus-1 and then gradually decreased to the level at diestrus on days 4–5. Thus, it is unlikely that blockade of the estrous cycle by neutrophil depletion was due to pseudopregnancy.

Ovarian production of E2 and P4 is regulated by LH and FSH [21,22], while LH and FSH production is regulated by E2 and P4 [23,24]. When the estrous cycle was blocked in this study, there were no histological changes in the ovary, suggesting that the levels of LH and FSH are also affected.

In conclusion, this study demonstrated that inhibition of neutrophil infiltration into the vaginal vault at metestrus led to blockade of the estrous cycle at diestrus presumably due to improper regulation of serum steroid hormone levels. Our findings may shed light on unexplained associations with ovarian dysfunctions and reduced fertility occurring mostly during active states of human inflammatory bowel disease [25,26], because in a rat model of severe colitis a significant decrease in uterine neutrophils is reportedly associated with estrous cycle disturbances [25]. Much work is required to elucidate how neutrophils regulate the serum levels of steroid hormones E2 and P4.

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