MICRO-ORGANISMS AND THE APPEARANCE OF LEUCOCYTES IN THE VAGINAL WALL OF CYCLIC FEMALE RATS AT METOESTRUS

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Summary. The present study was undertaken to examine whether leucocytic infiltration of the vagina of the rat at metoestrus is dependent on its contamination by micro-organisms. Observations were made on vaginal tissue that had been transplanted under the kidney capsule in cyclic rats, taking care to avoid infection during the transplantation procedure. In such grafts, changes occurred that were associated with ovulation and formation of the CL, but leucocytosis was never obtained at metoestrus. Cyclic changes were observed in the cell patterns of the vaginal smears of germ-free rats, and could be correlated exactly with the ovarian cycle. No leucocytes were present at metoestrus. Many micro-organisms were present in the vagina at pro-oestrus and oestrus in normal cyclic females, but not at metoestrus and dioestrus. It is concluded that the occurrence of leucocytosis in the vagina at metoestrus in normal cyclic female rats depends on the presence of micro-organisms.

INTRODUCTION

The appearance of leucocytes in the vaginal wall and subsequently in the vaginal lumen and vaginal smear, has been shown to be a reliable indicator of the early postovulatory period ('metoestrus') in several rodents, including the guinea-pig (Stockard & Papanicolaou, 1917), the rat (Long & Evans, 1922) and the mouse (Allen, 1922). The leucocytes appear 24 hr after the sudden decrease in oestrogen levels during the afternoon of pro-oestrus (Butcher *et al.*, 1974). The removal of oestrogenic stimulation results in a disintegration of the upper layers of the vaginal epithelium (Roig de Vargas-Linares & Burgos, 1965), associated with the irreversible differentiation of the basal epithelial layers (Barker & Walker, 1966). Early studies on the mechanism of vaginal leucocytosis led to controversial conclusions concerning the association of leucocytosis with the presence of micro-organisms in the vaginal lumen. Gardner & Allen (1937) studied leucocytosis of uterine epithelium following desquamation of cornified uterine epithelial cells; cornification had been obtained by the application of high doses of oestrogenic substances ('metaplasia'). It was observed that leucocytosis did not occur if the normal patency of the uterine lumen

to the outside world was obstructed by transplantation or ligation. It was thus concluded that contamination of the uterus with micro-organisms was a necessary requirement for the occurrence of leucocytosis. From experiments with male genital tract epithelium, however, Burrows (1935) concluded that micro-organisms were unlikely to be involved. Moreover, in his opinion, the regularity of leucocytosis in normal female rats was difficult to reconcile with vaginal micro-organisms as the cause of its occurrence. In agreement with the latter assumption, Beaver (1960) described leucocytosis of the vaginal wall in germ-free mice.

No definitive conclusion can be drawn from these studies since results obtained with uterine and male genital tract epithelium do not necessarily reflect the situation in the vaginal epithelium. In the study of Beaver (1960) quantitative differences may have been present in the leucocytic reactions in normal and germ-free mice. Moreover, direct observations of the vaginal microflora do not seem to have been made. It is possible that leucocytes are a normal constituent of the vaginal epithelium in certain circumstances, as has been described for the epithelium of the alimentary canal (Rovin *et al.*, 1966). It seemed of interest, therefore, to investigate whether micro-organisms were involved in vaginal leucocytosis at metoestrus in normal female rats. In the present study, the histology of the vaginal epithelium *in situ* was compared with that of vaginal epithelium that had been transplanted to a sterile or to a non-sterile environment. Vaginal cyclicity was also studied in germ-free rats, and direct observations were made on the vaginal microflora during the oestrous cycle.

MATERIALS AND METHODS

Experiment 1

The histology of vaginal tissue that had been grafted under the kidney capsule of (a) ten female rats with 5-day cycles, (b) neonatally castrated male rats bearing an ovarian graft, and (c) adult castrated male rats bearing an ovarian graft was studied. The animals were F₁ hybrids of two Wistar sub-strains, R and U, which were derived from the departmental colony. The animals were maintained under standardized lighting conditions (14 hr light/10 hr dark; light period from 05.00 to 19.00 hours) in a temperature-controlled room between 20 to 24°C, and were allowed free access to rat pellets (Hope Farms) and tap water. Ovarian and vaginal tissues for grafting were obtained from isogeneic prepubertal female rats which had a closed sterile vagina. Pieces (1 to 6 mm²) of vaginal tissue were placed under the kidney capsule. In male rats, a half vagina (cut transversely) was also placed between the abdominal skin and muscles. A surgical fistula in the vaginal graft was induced 2 or 3 weeks later to obtain a permanent 'vaginal opening' (van der Schoot & Zeilmaker, 1972). A whole ovary from a prepubertal donor was also implanted under the kidney capsule. Female rats and male rats castrated neonatally ovulated spontaneously. In fifteen male rats castrated as adults, ovulation and subsequent CL formation was induced by electrochemical stimulation (0.1 mA anodic d.c. for 60 to 120 sec) of the preoptic brain area, according to the method described by Everett & Radford (1961). Tissues were fixed in Bouin's solution and embedded

in paraffin wax. For histological study of ovarian, vaginal and brain tissue, 10- μ m sections were prepared; the sections were stained with haematoxylin and eosin.

Experiment 2

The vaginal and ovarian cycles were compared in ten germ-free and twenty conventional (kept under specific pathogen-free conditions) rats from the same breeding colony of the Organization for Applied Scientific Research (TNO-Zeist, The Netherlands). Vaginal smears were obtained from the rats under proven germ-free conditions (a) during an initial 2-week period, and (b) during a subsequent 1-week period. At the end of the second period, the animals were killed between 11.00 and 11.30 hours and ovarian and vaginal tissues were obtained for histological examination.

Experiment 3

(a) Microbiological studies were made of pairs of smears from ten 5-day cyclic RU female rats. Smears were stained both for microbiological (Gram) and cytological (Giemsa) examination. Each type of smear was assessed independently, and comparisons were made later. The contents of two pro-oestrous and six oestrous smears were cultured aerobically on blood agar and two oestrous smears were cultured anaerobically on a special medium (Wensinck & Ruseler-van Embden, 1971) in order to obtain more information concerning the kinds of micro-organisms present.

(b) In parallel with these experiments, crude estimations were made of changes in the pH of the vagina during the cycle. This was done by smearing the vaginal contents of all fourteen pro-oestrous, twelve oestrous, eleven metoestrous and twenty-five dioestrous rats onto different kinds of pH paper (universal pH paper and pH 7.2 to 9.7 paper: Machery, Nagel & Cy, Germany).

RESULTS

Experiment 1(a)

Histological study of vaginal grafts which had been removed from females at different stages of the ovarian cycle revealed the occurrence of cyclic changes in the appearance of cells shed from the vaginal epithelium. During late dioestrus, pro-oestrus and oestrus, cornified cells were shed in large numbers; during metoestrus and early dioestrus, few layers with nucleated cells appeared. All these cells remained between the kidney capsule and kidney parenchyma within a continuously growing 'balloon', filled mainly with vaginal epithelial cells. The wall of the balloon consisted of multilayered vaginal epithelium on top of vaginal stroma at the place where the vaginal transplant was present.

At other places, the inside of the wall of the balloon consisted of a single-layered vaginal epithelium with other (e.g. kidney) tissue at the outside. In the latter case, the vaginal epithelium, but not the vaginal stroma, had clearly extended from the vaginal transplant and grown along the whole inside of the balloon: except at the place of the transplant, there was no contact between vaginal epithelium and vaginal stroma. As can be seen in Pl. 1, Figs 1 and 2, there was no sign of leucocytic penetration of the multilayered vaginal epithelium during

any stage of the vaginal cycle. In order to investigate whether the small size of the vaginal transplants used in the above experiments might have been responsible for the absence of leucocytosis, the experiment was repeated in seventeen animals with nearly half a vagina grafted in two pieces under the kidney capsule. After three to five cycles of the vagina *in situ*, the histology of the vaginal grafts was studied. Again, no leucocytosis was observed during any stage of the cycle.

Experiment 1(b)

To study whether the grafted condition of the vaginal tissue was responsible for absence of leucocytosis, vaginal smears were studied in male rats, castrated neonatally, in which vaginal tissue was implanted under the abdominal wall and ovarian tissue under the kidney capsule. Metoestrus was characterized by massive penetration of leucocytes into the vaginal epithelium (Pl. 1, Fig. 3). Leucocytosis was absent in vaginal tissue transplanted under the kidney capsule in five similarly treated animals.

Experiment 1(c)

In most cases, when electrochemical stimulation was applied to male rats castrated as adults, in which ovarian and vaginal tissue (the latter both under the abdominal wall and the kidney capsule) had been implanted at least 4 weeks earlier, the histology of the ovarian grafts showed that the stimulus was adequate for the induction of CL. The continuous cornification observed in vaginal smears before the experiment was interrupted for several (3 to 6) days by leucocytic smears. The vaginal transplant under the kidney capsule, however, revealed the interruption of continuous cornification with a few layers of nucleated cells, without leucocytosis (Pl. 1, Fig. 4). Leucocytes also appeared in smears from animals in which the stimulus was inadequate for CL formation. In these animals, leucocytosis started about 24 hr after the application of the stimulus and continued for 2 to 5 days.

EXPLANATION OF PLATE 1

FIGS 1 and 2. Vaginal transplant under the kidney capsule of a cyclic female rat. The transplant was removed on the morning of metoestrus when nucleated cells were just beginning to be shed (B) from the vaginal epithelium (A). Previously, periods of shedding of cornified (D) cells had been alternating with those of shedding of nucleated cells (C).

FIG. 2. Detail of the right lower aspect of Fig. 1. This shows the absence of leucocytic infiltration into the vaginal epithelium at any stage of the ovarian cycle.

FIG. 3. Vaginal transplant with artificial vaginal opening from the abdominal wall of a male rat castrated neonatally and bearing an ovarian graft. The transplant was removed at metoestrus. The picture shows large numbers of leucocytes throughout the vaginal epithelium and in the 'vaginal lumen'.

FIG. 4. Vaginal transplant under the kidney capsule of a male rat castrated as an adult and bearing an ovarian graft. The transplant was removed 6 days after the successful induction of ovulation in the ovarian graft by electrochemical stimulation of the preoptic area. The continuous shedding of cornified cells from the vaginal epithelium in the period preceding ovulation (indicated by A) had been interrupted by the occurrence of few layers with nucleated epithelial cells (B) and followed again by constant cornification (C). No leucocytes were present within the layers of nucleated epithelial cells. D, vaginal epithelium; E, vaginal stroma.

PLATE 1

(Facing p. 64)

PLATE 2

FIGS 5 to 8. Vaginal smears and vaginal histology from germ-free rats at oestrus and metoestrus. During the period of study, all rats showed at least once a sequence lasting 2 days: the first day was characterized by the occurrence of cornified cells only (Fig. 5), the second day by the occurrence of many nucleated and vacuolated epithelial cells together with some cornified cells (Fig. 6). On the first day, the vaginal epithelium showed full development and the vaginal lumen appeared filled with cornified cells (Fig. 7). On the second day, the vaginal epithelium showed marked disintegration and the vaginal lumen appeared filled with nucleated epithelial cells (close to the epithelium) and cornified epithelial cells (central, in the lumen) (Fig. 8).

FIGS 9 and 10. Oil-immersion photographs of Gram-stained vaginal smears obtained from an oestrous (Fig. 9) and metoestrous (Fig. 10) rat. At oestrus, the vaginal smear shows lots of Gram-negative and Gram-positive micro-organisms (A) between cornified cells (B). At metoestrus, many leucocytes are present (large black dots) but no micro-organisms.

(Facing p. 65)

Experiment 2(a)

Vaginal smears were obtained from ten germ-free rats. It proved difficult to classify the vaginal smears; the classical findings of Long & Evans (1922) could not be applied to the smears taken from these rats. It was noticed, however, that all rats showed a sequence of two smears at least once. The first of these seemed to be an 'oestrous' smear, and this was followed by a smear that was full of nucleated and also non-nucleated cells (Pl. 2, Figs 5 and 6).

Experiment 2(b)

The germ-free rats were all killed between 11.00 and 11.30 hours. Serial sections of the ovaries revealed that one rat was in pro-oestrus at autopsy, two were in oestrus, three in metoestrus and four were in dioestrus. Sections of vaginal tissue from these animals showed some characteristic differences from the situation in normal ('conventional') rats. The epithelium of oestrous germ-free rats was greatly stratified and the vaginal lumen, unlike that of 'conventional' rats, was filled with cornified cells (Pl. 2, Fig. 7). The vaginal epithelium of germ-free rats during metoestrus was devoid of leucocytes, and the luminal cells were nucleated. The lumen itself contained large masses of cornified and nucleated epithelial cells (Pl. 2, Fig. 8). 'Conventional' rats showed few intact epithelial cells, but leucocytes were present in the vaginal lumen and leucocytic penetration occurred throughout the epithelium. It appeared that vaginal epithelial cells in germ-free rats could remain in the vaginal lumen for some time.

Experiment 3(a)

Comparison of cytological and bacteriological data revealed that all 36 smears from pro-oestrous and oestrous rats contained many Gram-positive and Gram-negative micro-organisms (Pl. 2, Fig. 9). By contrast, only 2/20 metoestrous smears and 0/40 dioestrous smears contained bacteria. Aerobic culture revealed the presence of *Escherichia coli*, *Klebsiella* and other organisms. Anaerobic culture revealed no micro-organisms other than those present in the aerobic cultures. Marked changes apparently occurred in the number of micro-organisms present in the vagina during the cycle, and the appearance of leucocytes at metoestrus coincided with disappearance of micro-organisms.

Experiment 3(b)

Smears from pro-oestrous and oestrous rats revealed pH values between 7 and 8 on both kinds of pH paper. By contrast, leucocytic smears from metoestrous rats and leucocytic or mucous smears from dioestrous rats revealed pH values that were higher than 9. Cyclical fluctuations occurred in the pH of vaginal smears with consistently high values during metoestrus and dioestrus.

DISCUSSION

The present experiments indicate that leucocytosis of the vagina in rats at metoestrus is dependent on the presence of micro-organisms in the vagina. The leucocytic reaction may thus be considered as an inflammatory reaction of the

vaginal wall from irritants in the vaginal lumen. It may be assumed that these irritants are associated with the micro-organisms that are present in large numbers during pro-oestrus and oestrus. It follows that, at pro-oestrus and oestrus, an effective protection against these bacterial irritants is present since during that period leucocytes do not appear in the vaginal wall or lumen. The moment of leucocytosis coincides with the time of maximal epithelial breakdown in the vagina due to decreased oestrogenic stimulation, i.e. at metoestrus (Barker & Walker, 1966). The leucotoxic factors in the vaginal lumen can apparently reach the sub-epithelial vaginal layers only in these circumstances. After metoestrus, a mucous vaginal epithelium starts to develop under the influence of the change in hormonal stimulation (Barker & Walker, 1966) and leucocytosis stops. A high pH, as observed here, may minimize the possibilities for active microbial life (Hynes, 1964).

It follows that three mechanisms are active in the rat to prevent infection from the vagina: (1) the mechanical barrier of mucus and/or cornified cells during pro-oestrus and oestrus, (2) the action of leucocytes at metoestrus, and (3) mucification combined with high pH during the dioestrous period. Of these, the regular elimination of micro-organisms by leucocytes might be specifically important for the prevention of uterine infection: it is well-known that uterine infections occur frequently in circumstances of continuous exposure to oestrogenic hormones, either by exogenous application (Weinstein *et al.*, 1937) or by endogenous production (Everett, 1939).

An important argument in favour of the concept of leucocytosis as a reaction to micro-organisms in the vaginal lumen was obtained in experiments with germ-free rats. Such animals might not show a normal pattern of leucocytosis in response to leucotoxic agents: indeed, the mobilization rate of leucocytes in germ-free animals seems lower than in 'conventional' animals (Luckey, 1963). However, Parr *et al.* (1967) showed that the leucocytic reaction of the uterus to a silk suture in the lumen of germ-free rats was only quantitatively different from the reaction in conventional rats, but qualitatively similar in its histological aspects and in its local prevention of implantation. Also, Beaver (1960) showed that leucocytosis of the vaginal wall could be obtained in germ-free mice in response to treatment with progesterone. The present results with germ-free rats thus differ from those obtained by Beaver (1960) with mice. Leucocytosis at metoestrus in rats perhaps differs in some characteristics from leucocytosis in mice: comparison of pictures of leucocytosis of the vaginal wall in rats (Long & Evans, 1922) and mice (Allen, 1922) reveals differences in the numbers of leucocytes, in the timing of leucocytic penetration and the preferential localization. Experiments are in progress to analyse these differences in more detail. In the experiment with male rats castrated as adults, it was observed that leucocytosis occurred in vaginal smears without preceding ovulation or CL formation in the ovarian graft. Several reports in the literature mention the occurrence of leucocytic vaginal smears without preceding ovulation (Everett, 1939; Bradbury, 1941; Mandl, 1961; Stern *et al.*, 1965). It seems worthwhile to test whether an association between decrease of oestrogens and leucocytosis can be substantiated.

The technique of transplanting vaginal tissue into a sterile environment in

order to study ovarian activity has been used previously (Yazaki, 1959; Zeilmaker, 1963; Kawashima, 1964; Flerko *et al.*, 1967; van der Schoot & Zeilmaker, 1972). These authors demonstrated a relationship between the changes in the pattern of cells shed from the vaginal epithelium and changes in hormonal stimulation; indirectly, they confirmed the principle of 'irreversible differentiation' of the basal layers of the vaginal epithelium under the influence of a certain type of hormonal stimulation, as established by Barker & Walker (1966). The present results reveal some new characteristics of these vaginal grafts. When the graft was removed on the day of metoestrus at noon, intact nucleated cells were only just beginning to replace the cornified cells shed from the vaginal transplant (Pl. 1, Figs 1 and 2). The intact vagina of 'conventional' and germ-free (Pl. 2, Fig. 8) rats had already lost a large number of layers with nucleated epithelial cells showing signs of autolysis. Cornification appears to continue for much longer in the vaginal transplant than in the intact vagina. Micro-organisms are not involved since the phenomenon in the intact vagina also occurs in germ-free animals. The difference might be due to the fact that the vaginal epithelial cells become deprived of energy sources and autolyse after destruction of intercellular contacts in the intact vagina early in oestrus; by contrast, contacts with environmental cells in the vaginal transplant are maintained so that energy supplies continue and the cells can develop to the stage of cornification. Vaginal epithelium in contact with the underlying vaginal tissue showed cyclical changes under the influence of changes in the hormonal environment. By contrast, vaginal epithelium not in contact with the vaginal stroma remained as a single layer, resembling that of ovariectomized female rats. This observation suggests that the vaginal epithelium needs contact with the underlying vaginal tissue to undergo cyclical changes, and may indicate that the primary site of action of ovarian hormones affecting the vaginal epithelium lies outside the epithelial cells, probably in the vaginal tissue under the epithelium.

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