Relationship of Vaginal Cytology to Alterations of the Vaginal Microflora of Rats During the Estrous Cycle

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Received for publication 8 September 1976

The influence of cyclic changes occurring in the vaginal tract during the estrous cycle upon the indigenous microflora of the vagina has been investigated by semiquantitative techniques in virgin female rats. By plate counts performed on material lavaged daily from the vaginal tract of several rats, it is apparent that bacterial counts are elevated in the proestrus and estrus phases of the cycle to values several orders of magnitude greater than those observed during metestrus or diestrus phases. Increases in vaginal bacterial counts were associated with the presence of cornified epithelial cells in the vagina; these cells were predominantly nonviable. Decreases in the vaginal bacterial content were related to the influx of leukocytes into the vagina after estrus. When leukocytes were present in the vaginal tract, they were 90 to 100% viable. From these observations it has been concluded that the female vaginal tract and the bacteria which colonize it represent a dynamic ecosystem which is responsive to cyclic events occurring in the estrous cycle. The changing cellular content of the vaginal tract may have relevance to the observed cyclic changes in the bacterial content of the vagina.

For many years students of the indigenous microflora of the human vagina have recognized that vaginal microflora is altered by the onset of puberty, pregnancy, and menopause (1, 2, 4, 9), but whether the menstrual cycle has any similar effect has not been studied. In our laboratory the vaginal tract of the virgin rat has been found to be a useful model for studying the relationship of the estrous cycle to colonization. Preliminary studies have suggested that, in the rat model, events in the estrous cycle profoundly affect the vaginal bacterial population. This report describes studies which suggest the relationship of cytohormonal changes in the vaginal tract to changes in bacterial content.

MATERIALS AND METHODS

Animals used in this study were randomly bred, virgin female rats obtained from Small Animal Supply Co., Omaha, Neb. Rats were kept in stainless-steel cages with an ample layer of commercial bedding. Food and water were allowed ad libitum, and light (12 hr dark and 12 hr light) and temperature were electronically controlled.

Cytological evaluation of the vaginal contents employed microscopic examination of unstained smears. Quantitative cytological evaluation involved determination of the relative numbers of each of the three types of vaginal cells in a hemocytometer. Cells observed in the rat vagina include polymorphonuclear leukocytes, noncornified epithelial cells, and cornified squamous epithelial cells. The relationship of each of these cell types to the stage of the estrous cycle has been described (8).

Vaginal lavage involved aspiration of sterile normal saline into a sterile medicine dropper. The tip of the medicine dropper was inserted a few millimeters into the vagina of a restrained rat and diluent was intermittently expressed and reaspirated several times.

Enumeration of bacteria obtained from the rat vaginal tract employed blood agar as a plating medium. This medium was suitable for growth of organisms which were isolated from the rat vaginal tract (5). Saline for vaginal lavage was aspirated from a 5-ml dilution blank; lavage was performed as described, and the aspirate was returned to the dilution blank. Logarithmic dilutions of the lavage material were made in saline, and 0.1 ml of each dilution was spread on blood agar. In this study duplicate platings were not done, although previously it was shown that the standard error of the mean for multiple platings of vaginal lavage material was about 10% (6). Incubation was for 24-48 hr at 37°C in an anaerobic chamber (Coy Manufacturing, Ann Arbor, Mich.) with a gas phase of approximately 90% N₂, 5% H₂, and 5% CO₂. The longer period of incubation did not increase colony counts.

RESULTS

Examination of Gram-stained vaginal smears has suggested that the bacterial content of the genital tract varies predictably throughout the estrous cycle (B. Larsen, A. J. Markov-
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To further validate the lavage technique for quantitating the vaginal flora, the following study was done. Vaginal lavage was performed on 11 rats, and the material recovered was diluted and plated. Each rat was subsequently sacrificed, and the cervix and vagina were excised and macerated with sterile saline in a Potter-Elvejem glass tissue homogenizer. The homogenate was diluted and plated to determine the number of culturable bacterial counts which were not recoverable by vaginal lavage. As seen in Fig. 1, the lavage-plate count technique was a highly reliable indicator of the bacterial content of the vaginal tract in that at least 90% of all colony-forming units which could be cultured by the media and other conditions of incubation were recovered by lavage, leaving less than 10% associated with the tissue.

Because of the demonstrated effectiveness of the lavage-plate count technique, it was employed to establish a relationship between vaginal bacterial counts and stage of the estrous cycle. Cytological evaluation of the lavage material established the stage of the estrous cycle. Initially the relationship was studied in 6 rats and subsequently in an additional 24 rats. Data were consistent with the results obtained from two rats illustrated in Fig. 2. The following generalizations should be noted. Bacterial counts from the vaginal tract of rats varied significantly and cyclically during the estrous cycle. Bacterial counts were elevated every fourth day, corresponding to the length of the rat estrous cycle. The highest observed bacterial counts occurred coincidentally with the estrus phase of the cycle. Similar cyclicity of vaginal bacterial content has been observed in numerous other rats.

Because the bacteriological changes and changes in vaginal cytology were seen together, the relationship of the cytological to bacteriological content of the vagina was noted in the following study. Four rats having normal estrous cycles were examined every 12 h for 12 days by vaginal lavage. Plate counts were performed, and a portion of the lavage material was placed in a hemocytometer for quantitative cytology. Figure 3 shows the relationship of the three types of cells to the number of vaginal bacteria in one of the four rats. It appears (Fig. 3) that elevated bacterial counts correspond to high percentages of cornified epithelial cells, but that bacterial counts began to increase when noncornified epithelial cells were the predominant type. Bacterial counts appeared to be inversely related to leukocyte counts.

Data obtained from the four rats for the 12 days of the study were pooled, and regression analysis (3) was performed. Figure 4 shows the least-squares lines relating bacterial counts to each of the three cell types. Also included in these analyses was the relationship of the combined frequency of cornified and noncornified epithelial cells (described as epithelioid cells). As shown by Fig. 4, the greatest response of bacterial population to single cell types was seen with cornified cells or leukocytes. The data did not suggest that noncornified epithelial cells were related to increasing bacterial counts which were observed during the proestrus phase of the cycle, nor did the combined frequencies of the two epithelial cell types show any greater effect on bacterial counts than did...
cornified cells alone (slope = 0.02482 for cornified cells, and slope = 0.02241 for "epithelioid" cells).

The strength of correlation and associated significance is shown in Table 1. These data are consistent with the concept that the cytological nature of the genital tract may control colonization. To better understand how the cells of the vagina may influence the bacterial content of the vagina, the ability of each cell type to exclude trypan blue was noted in each of five rats during each stage of the estrous cycle. Results (Table 2) indicate that cornified cells are consistently nonviable whereas the other two cell types are usually viable.

**DISCUSSION**

The purpose of this investigation has been to determine if the bacterial content of the vaginal tract is influenced by the estrous cycle. Although in the case of humans (1, 2, 4, 9) ovarian function has been related to changes in bacterial colonization (primarily with respect to the presence or absence of lactobacilli), flora changes during the menstrual cycle are not well documented. Because menstrual and estrous cycles and their component phases occur with relative rapidity (lasting only days as compared to stages of life such as prepuberty and menopause which last for years), it may be anticipated that no changes in the vaginal microflora should be observable during these brief cycles. However, this study has demonstrated that quantitatively the density of vaginal bacterial colonization in the female rat varies profoundly and cyclically during the estrous cycle. A particularly striking aspect of the data presented is that changes in the bacterial content in the animal model studied amount to several orders of magnitude and occur over the time span of 1 day.

It should be noted that the method employed for enumerating bacteria present in the vaginal tract of rats was somewhat selective in that not all bacteria were removed from the vaginal tract by lavage, and the plating medium (sheep blood agar) could not be expected to support growth of all organisms which theoretically might be encountered. However, this medium is suitable for cultivation of the bacterial species we have identified in the vaginal tract of the type of rats used in the present study (5), and all morphological types seen in Gram-stained vaginal smears can be accounted for by culture techniques used in this and previous studies (Larsen et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, B82, p. 25). In addition, we have also previously shown that vaginal counts done by Gram-stained vaginal smear show cyclic changes similar to those seen when counts were done by the lavage-plate count technique. These facts suggest that the method

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**Fig. 2. Vaginal lavage followed by plate count of the material recovered from the vagina by lavage was performed daily on two rats for 12 days. The results of the plate counts, expressed as colony-forming units (CFU) recovered from each rat, are plotted on the ordinate with the day of the study on the abscissa. Counts from one of the rats is represented by crosses, counts from the other are represented by circles. Results of cytological evaluation are represented by the boldface letters near each data point. Abbreviations: e, estrus; m, diestrus; d, diestrus; p, proestrus.**
Plate counts on material lavaged from the vagina were performed twice daily for 12 days on four rats. Hemocytometer counts of the frequencies of cornified, noncornified epithelial, and leukocytic cells in the vaginal lavage were also determined. These data are for one of the four rats and, as in the previous figure, correlate vaginal bacterial counts with the day of the study (upper graph). The percentage of each cell type in the material lavaged from the vagina is presented in the lower three graphs.
we used to measure vaginal bacterial content was reasonably reliable. The reliability of the lavage method was further demonstrated by the finding that greater than 90% of the cultivable vaginal bacteria was recovered by vaginal lavage. These data also incidentally suggest that the vaginal bacteria were probably situated superficially on the epithelium since greater than 90% were accessible and elutable by vaginal lavage. Determination of the particular species involved in the cyclic fluctuations of the vaginal flora during the estrous cycle was not a part of this study but will certainly be an important aspect of future work.

It is appropriate, however, to consider how

**TABLE 1. Relationship of vaginal cytology to vaginal bacterial content**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Regression analysis</th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R²</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td>Epithelial</td>
<td>0.02197</td>
<td>0.08659</td>
<td></td>
</tr>
<tr>
<td>Cornified</td>
<td>0.36311</td>
<td>0.00001</td>
<td></td>
</tr>
<tr>
<td>Leukocytes</td>
<td>0.27813</td>
<td>0.00001</td>
<td></td>
</tr>
<tr>
<td>Epithelioid</td>
<td>0.26887</td>
<td>0.00001</td>
<td></td>
</tr>
</tbody>
</table>

**TABLE 2. Trypan blue exclusion from various cell types throughout the estrous cycle**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Percent of cells of specified type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Estrus*</td>
</tr>
<tr>
<td>Cornified cells</td>
<td>19.8</td>
</tr>
<tr>
<td>Epithelial cells</td>
<td>100</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>80.0</td>
</tr>
</tbody>
</table>

* Stage of the cycle.
vaginal lavage itself may affect the cytological and bacteriological parameters studied. Vaginal cytology is a reflection of ovarian function and continues as expected despite daily or twice-daily vaginal lavage. The daily changes in vaginal cytology done by vaginal lavage show no differences with respect to cellular types or appearance of the cells when compared to the cytological evaluations based on the more gentle procedure of gathering cellular material with a wire loop. Therefore, vaginal lavage probably does not appreciably affect vaginal cytology.

It is possible that vaginal lavage could have influenced the vaginal bacterial content either with respect to bacterial counts or species present. Because this study did not address the question of how individual bacterial species vary during the cycle, the possibility of species alterations as a result of the lavage procedure cannot be judged. However, it is possible to determine from these studies that probably little quantitative perturbation of the flora resulted from the lavage procedure. First, we failed to find any differences between the cyclic variations in bacterial counts in rats receiving vaginal lavage daily as compared to those receiving lavage twice daily. In both cases peak counts were observed at 4-day intervals followed by 2 days of significantly lower counts. At the end of 12 days the cyclic variation in rats lavaged twice daily showed as much stability in the pattern of bacterial counts as during the first days of the study. Second, vaginal lavage appeared not to prevent bacterial counts from reaching expected peak levels, nor was the rate of increase in bacterial counts detectably affected even when lavage was done twice daily. It may seem that, if lavage removed 90% of the vaginal bacteria, the numbers obtained by lavage at some later time may be depressed as a result. However, if 10% of the original vaginal bacterial population remains, fewer than four divisions will be required to repopulate the vagina with the original number of bacteria. Therefore, an in vivo generation time of as much as 3 h could be characteristic of all vaginal organisms and still allow repopulation within 12 h after vaginal lavage.

The cyclic variation in the bacterial content of the vaginal tract immediately evokes the question of the mechanism of this pattern of flora control. Since the most apparent changes in the intravaginal environment were changes in the exfoliative cytology, we examined the relationship between the host cells and dynamics of the vaginal bacterial population. From the data presented it appeared that if the vaginal bacterial population is influenced by vaginal cellular composition, the cells most likely involved are cornified squamous epithelial cells, enhancing colonization, and leukocytes, diminishing colonization. As expected, trypan blue dye exclusion from cornified cells was seen in a small percentage of cells of this type which suggested that these were effete cells and may therefore be undergoing a process of degeneration with release of nutrient materials into the intravaginal environment. This process could then account for the expansion of the vaginal bacterial population described. However, detracting from this explanation is the fact that bacterial counts were observed to increase during the proestrus phase of the cycle when only minimal numbers of cornified cells were present. The trypan blue study also indicated that the leukocytes in the vaginal tract are mostly viable cells and because of their well known phagocytic function may help to explain why bacterial counts decrease during the metestrus phase of the cycle. This phase is characterized by an influx of leukocytes and the disappearance of the cornified cells.

Although it is possible to tentatively explain the population dynamics of the vaginal ecosystem on the basis of cytology, some caution is necessary since the cytological correlation with bacterial counts revealed that linear regression accounted for only 36% of the data points for cornified cells and only 28% of the data points for leukocytes. The data unexplained by linear regression may be attributed to biovariation, experimental error, and may also indicate that the cellular content of the vagina per se may not be the factor controlling the vaginal microflora, but may be indicative of other conditions or substances prevailing in the vagina concomitantly with the cornified and leukocytic cells. Other factors which may be important in the cyclic fluctuation of vaginal counts include pH, redox potential, presence of mucin, vaginal blood supply, and the presence and quantity of antibacterial substances. Further studies will be required to fully evaluate the relationships preliminarily studied and reported here.

Although the data presented involves only studies of rat vaginal flora, the relevance of these data to other species should be considered. For example, it is well known that in the absence of ovarian hormones, Neisseria gonorrhoeae may infect the human vaginal mucosa, but if the vaginal epithelium is matured under estrogen influence, gonorrhoea becomes a cervical rather than vaginal infection (7). The acquisition of other vaginal infectious diseases may depend on the cytological and hormonal status of the genital tract although this possibility has not been explored.
In conclusion, the present study represents a description of control exerted on the flora of the female vaginal tract by normal physiological parameters. The development of the rat as an animal model for the study of the genital flora may ultimately, significantly enhance our understanding of factors which promote and limit the filling of living ecological niches with bacteria.

LITERATURE CITED