INFECTION AND IMMUNITY, June 1999, p. 3135–3140 Vol. 67, No. 6
0019-9567/99/$04.00+0

Analysis of Vaginal Cell Populations during Experimental Vaginal Candidiasis

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Received 16 October 1998/Returned for modification 6 December 1998/Accepted 20 February 1999

Studies with an estrogen-dependent murine model of vaginal candidiasis suggest that local cell-mediated immunity (CMI) is more important than systemic CMI for protection against vaginitis. The present study, however, showed that, compared to uninfected mice, little to no change in the percentage or types of vaginal T cells occurred during a primary vaginal infection or during a secondary vaginal infection where partial protection was observed. Furthermore, depletion of polymorphonuclear leukocytes (PMN) had no effect on infection in the presence or absence of pseudoestrus. These results indicate a lack of demonstrable effects by systemic CMI or PMN against vaginitis and suggest that if local T cells are important, they are functioning without showing significant increases in numbers within the vaginal mucosa during infection.

Recurrent vulvovaginal candidiasis (RVVC) is a significant problem in otherwise healthy women of childbearing age (24, 42, 43). Since no exogenous predisposing factors such as pregnancy, use of oral contraceptives or antibiotics, or diabetes mellitus are known to influence the incidence of RVVC, it has been postulated that some form of immune deficiency or dysfunction is responsible for recurrent episodes (more than three per year) of vaginitis (16, 42, 45). *Candida albicans*, a commensal organism of the intestinal and reproductive tracts, is the causative agent in most cases of RVVC (42). Since cell-mediated immunity (CMI), through the function of T cells and cytokines and specifically through a Th1-type response, is the predominant host defense mechanisms against *C. albicans* infections of other mucosal tissues (5, 31, 40, 41), we have been examining CMI-type host defense mechanisms against *C. albicans* at the vaginal mucosa. Our studies have been both clinical, using women with RVVC (9, 11), and experimental, using an estrogen-dependent murine model of vaginal candidiasis (8, 10, 12–15). To date, our studies suggest that *Candida*-specific Th1-type CMI in women with RVVC, as well as that induced in the peripheral blood and/or secondary lymphoid tissues (i.e., lymph nodes) of mice as a result of vaginal exposure to *C. albicans*, does not provide protection against vaginal candidiasis (10, 11, 13–15). On the basis of these observations, we postulated that CMI induced at the vaginal site is important for protection against vaginitis (16). Studies were therefore initiated to examine the presence and phenotype of T cells at the vaginal mucosa of naive mice. Interestingly, we and others have found that vaginal T cells are phenotypically distinct from T cells in the periphery (18–20, 30), supporting the concept of immunological independence or compartmentalization at the vaginal mucosa. Specifically, we have noted in vaginal tissue a higher percentage of γδ T-cell receptor (TCR)-positive cells, low or undetectable levels of $CD8^+$ cells, and an atypical expression of the CD4 protein on $CD4^+$ T cells under nondenaturing conditions (18). The latter was shown by the inability of GK 1.5 but not 2B6 anti-CD4 antibodies to recognize vaginal $CD4^+$ cells when detected by flow cytometry following dual staining with the two antibodies on lymph node cells tested separately or when added to the vaginal-cell preparation. However, under denaturing conditions, as shown by immunohistochemistry, vaginal cells could be recognized by GK 1.5 anti-CD4 antibodies, providing evidence that the vaginal CD4 protein was conformationally distinct from that found on systemic $CD4^+$ cells (46). The atypical expression of the CD4 protein on vaginal T cells was extended as well to the mRNA level. Despite the presence of sufficient numbers of vaginal $CD4^+$ T cells, vaginal CD4 mRNA could be detected consistently only by using a high-efficiency *Taq* DNA polymerase in reverse transcription-PCR (RT-PCR) and the CD4 mRNA was absent in a purified population of vaginal cells that atypically expressed the CD4 protein. From these data, we postulated that vaginal $CD4^+$ cells express a unique CD4 mRNA and that any detectable CD4 mRNA in such reactions represented low-level systemic cell contamination within the vaginal mucosa (46). Thus, vaginal and systemically derived $CD4^+$ T cells can be distinguished at both the protein and molecular levels, providing the means to identify and study each within the vagina under various experimental conditions.

Polymorphonuclear leukocytes (PMN) are an important innate host defense mechanism against *C. albicans* in the systemic circulation (31, 47) and have significant anti-*Candida* activity in vitro (7, 29). PMN are often observed in the vagina during an experimental infection in mice, but their presence does not seem to correlate with a reduction in the fungal titers in the vaginas of infected animals, calling into question their role in host defense against *C. albicans* at that site.

The purpose of this study was to evaluate changes in murine vaginal T-cell populations as well as the effects of the depletion of PMN on primary infection in the presence or absence of pseudoestrus. T-cell populations were also assessed following secondary vaginal challenge where partial protection occurs (10).

Analysis of vaginal T cells during a primary *C. albicans* vaginal infection. Untreated CBA/J mice ($H\text{-}2^k$, 8 to 10 weeks of age; purchased from the National Cancer Institute, Frederick, Md.) or mice treated with 0.02 mg of estradiol valerate dissolved in sesame oil to induce a state of pseudoestrus were inoculated intravaginally with *C. albicans* 3153A (5×10^4 blas-

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TABLE 1. Flow cytometric analysis of vaginal T cells during primary *C. albicans* vaginal infection[a]

Cell type	% of positive cells[b] after following treatment:											
	Estrogen and no infection				Estrogen and infection				Infection and no estrogen			
	1	2	3	5	1	2	3	5	1	2	3	5
αβ TCR^+ $CD4^+$	40	29	32	35	42	28	30	32	39	30	23	29
γδ TCR^+ $CD4^+$	20	19	20	21	22	16	21	19	23	20	19	17
γδ TCR^+ $CD4^-$	12	3	7	4	13	1	5	6	15	0.2	0.7	2
CD4/CD8	36/5	33/3	40/3	35/3	36/3	50/2	39/3	37/5	43/8	35/5	55/1	37/3
$2B6^+GK1.5^-$	34	39	38	40	38	41	36	39	41	33	42	37
$2B6^+GK1.5^+$	5	3	4	3	5	2	5	2	3	4	6	2

[a] Data presented from a single experiment of two repeats with pooled cells (10^5/sample) from 9 to 10 mice.
[b] Based on lymphoid cell-like limits of cells collected from collagenase-digested vaginas. The numbers 1, 2, 3, and 5 for each treatment refer to weeks postinfection.

toconidia) (12, 17). Controls included estrogen-treated animals given phosphate-buffered saline intravaginally. Over a period of 5 weeks, groups of 10 to 15 animals were assessed for their vaginal fungal burden by quantitative culture of vaginal lavage fluid (12), and extracted vaginal lymphocytes (enzymatic digestion) (15) and whole tissue were assessed for T-cell phenotypes by flow cytometry (18), immunohistochemistry (46), or RT-PCR (46). In three separate experiments, the vaginal fungal burden in mice infected in the presence or absence of pseudoestrus was similar to that observed previously (13); i.e., the mice were persistently infected (>5 weeks) with high fungal titers (10^4 to 10^5 CFU) under pseudoestrus conditions, while short-lived (<3 weeks) infections with lower fungal titers (10^1 to 10^4 CFU) occurred in the absence of pseudoestrus. *C. albicans* was not detected in estrogen-treated uninfected mice (data not shown).

Flow cytometric analysis of the vaginal lymphoid cells (~10^5) from one of two trials performed in which fluorochrome-conjugated anti-CD3, anti-CD4 (2B6 or GK 1.5), anti-CD8, anti-TCRαβ, and anti-TCRγδ antibodies (18) (PharMingen Corp., San Diego, Calif.) were used is summarized in Table 1. There were no significant changes in the percentages of vaginal αβ or γδ TCR^+ cells or $CD4^+$ or $CD8^+$ subpopulations of cells in estrogen-treated and untreated infected mice compared to uninfected mice throughout 5 weeks of infection. This included both vagina-specific $CD4^+$ cells that atypically express the CD4 protein ($2B6^+$ GK 1.5^-) and $CD4^+$ cells of systemic origin ($2B6^+$ GK 1.5^+). Isotype control antibodies showed negligible staining (data not shown). Similar results were observed in a second trial (data not shown), and neither trial revealed any distinct pattern of change in absolute T-cell numbers between groups of animals. Similarly, immunohistochemical staining for T cells in infected and uninfected vaginal tissue by using purified antibodies with the same T-cell specificities detected by the avidin-biotin-peroxidase system (Vector Laboratories, Burlingame, Calif.) showed no evidence of changes in numbers of vaginal T cells as a result of infection (data not shown).

Semiquantitative RT-PCR was used as a more sensitive measure of T-cell expression in the vagina during infection. For this, total RNA was extracted from vaginal or lymph node tissue and first-strand cDNA was immediately synthesized from 1 μg of total RNA with oligo(dT) primers and avian myeloblastosis virus reverse transcriptase (Promega, Madison, Wis.). Primer sets derived from systemic cell cDNA sequences for CD3 (36), CD4 (Clontech, Palo Alto, Calif.), CD8 (Clontech), TCR-β chain (1), TCR-δ chain (26), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (21) as the housekeeping gene were synthesized at the Louisiana State University Core Laboratories, New Orleans, La. Traditional *Taq* DNA polymerase (Promega) was used in most PCR amplifications, but high-efficiency (HE) *Taq* DNA polymerase (Platinum *Taq*) (GIBCO, Grand Island, N.Y.) was used specifically for CD4 PCR amplifications from vaginal tissue (46). Negative controls included PCR amplifications in the absence of cDNA. The PCR products were separated by electrophoresis and visualized by ethidium bromide staining. For semiquantitative analyses, preliminary dilutional studies were conducted to optimize the concentration of cDNA required for each primer set, ensuring that the kinetic interpretation would not be hindered by saturation of primer sets with cDNA. Any change in levels of mRNA (cDNA) over time was expressed as a ratio of amplified cDNA product from a specific primer set to GAPDH (measured in pixel intensities by a video capture gel documentation system 1000 [BioRad, Richmond, Calif.]). Figure 1A shows the cDNA amplification corresponding to several T-cell surface markers (CD3, CD4, CD8, TCR-β, and TCR-δ) and the housekeeping gene (GAPDH) in lymph node and vaginal tissue by RT-PCR with regular *Taq* DNA polymerase; it also shows CD4 cDNA amplified by HE *Taq* DNA polymerase. Negative controls without cDNA showed no amplification products (data not shown). In the semiquantitative RT-PCR analyses throughout the 5-week infection period (Fig. 1B), there were no distinctive changes in mRNA expression of CD3, putative systemically derived CD4 (with HE *Taq*), TCR-β chain, or TCR-δ chain from estrogen-treated or nontreated infected mice compared to uninfected control mice. No amplification of CD4 or CD8 cDNA with regular *Taq* DNA polymerase was observed throughout the 5-week period (data not shown).

These results suggest that despite the immunocompetent potential of the vaginal mucosa (32), no detectable changes in vaginal αβ or γδ T cells occurred during a primary vaginal *C. albicans* infection in the presence or absence of estrogen. Furthermore, the lack of increases in the numbers of GK 1.5^+ $CD4^+$ T cells or of detectable CD4 mRNA when regular *Taq* DNA polymerase was used during the infection suggests that systemically-derived $CD4^+$ T cells did not infiltrate into the tissue in response to the infection. Another interesting observation was the lack of effect of estrogen on the numbers or percentages of resident T cells in the vaginal mucosa of infected or uninfected mice. Although estrogen inhibits cellular immunity (25, 27, 28), these data suggest that the resolution of infection in the absence of estrogen may have more to do with the ability of the organism to adhere to tissue than to any putative T-cell immune function. Alternatively, estrogen may have a negative effect on other immune system parameters (e.g., antibody production) that may be important in the host defense against vaginitis (4, 6, 37).

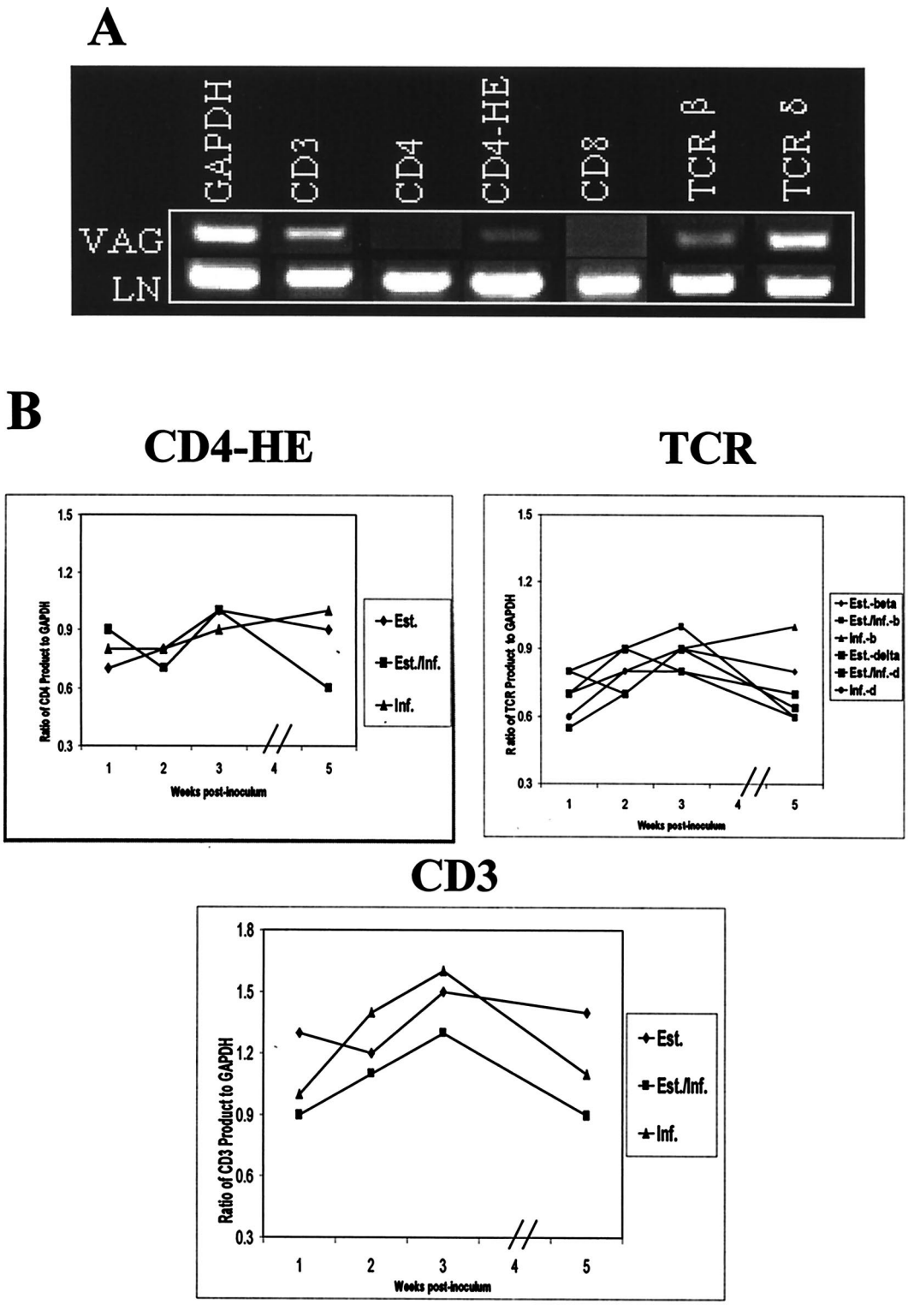

FIG. 1. RT-PCR of T-cell surface marker mRNA expression during primary *C. albicans* vaginal infection. Total RNA extracted from vaginal tissue or lymph nodes (three mice per group) was subjected to RT-PCR with primers specific for CD3, CD4, CD8, TCR-β constant region, and TCR-δ constant region of systemically derived T cells, using normal or HE *Taq* DNA polymerase. GAPDH served as the housekeeping gene. (A) RT-PCR of T-cell surface marker mRNA expression in lymph nodes and vaginal tissue of naive mice. (B) Semiquantitative RT-PCR of CD4, TCR-β, TCR-δ, and CD3 during a primary vaginal *C. albicans* infection. Results over time are expressed as the ratio of each product in pixel intensities to that for GAPDH. Est., estrogen-treated uninfected mice; Est./Inf., estrogen-treated infected mice; Inf., non-estrogen-treated infected mice; beta (b), β-chain; delta (d), δ-chain. The experiment was repeated twice, and the results shown are representative.

TABLE 2. Flow cytometric analysis of vaginal T cells following primary or secondary *C. albicans* vaginal infection[a]

Cell type:	% of positive cells[b] on:			
	Day 4 after:		Day 10 after:	
	Primary infection	Secondary infection	Primary infection	Secondary infection
αβ TCR^+ $CD4^+$	31	36	35	36
γδ TCR^+ $CD4^+$	14	12	15	13
γδ TCR^+ $CD4^-$	3	2	4	3
CD4/CD8	37/2	48/2	33/3	48/3
$2B6^+GK1.5^-$	46	45	43	46
$2B6^+GK1.5^+$	8	5	6	7

[a] Data presented from a single experiment of two repeats with pooled cells (10^5/sample) from 9 to 10 estrogen-treated primary or secondary infected mice.
[b] Based on lymphoid cell-like limits of cells collected from collagenase-digested vaginas.

Analysis of vaginal T cells during a secondary *C. albicans* vaginal infection. A similar analysis of T-cell expression was conducted during a secondary *Candida* vaginal infection, where partial protection has been observed previously (10). For this, animals were inoculated with 5×10^5 stationary-phase *C. albicans* blastoconidia in the absence of estrogen. After 4 weeks (following spontaneous resolution of the primary infection), the animals were treated with estrogen as above, and 72 h later, they were inoculated a second time with 5×10^4 *C. albicans* blastoconidia. In two experiments, a significant reduction in the vaginal fungal burden was observed on both days 4 and 10 after secondary inoculation compared to that in estrogen-treated mice given a primary infection ($P < 0.002$) (data not shown), consistent with the partial protection reported previously (10). Flow cytometric analysis on extracted vaginal lymphocytes (Table 2), however, showed no changes in the various T-cell subsets between mice with primary and secondary infections through 10 days, including the atypical expression of the CD4 cells as assessed by the two epitope-distinct anti-CD4 antibodies. Similarly, no differences were observed in vaginal CD3, CD4, TCR-β, and TCR-δ cells between mice with primary and secondary infections as assayed by immunohistochemistry or RT-PCR of tissue-derived mRNA (data not shown). Thus, if the local T-cell compartment is responsible for the partial protection against a second vaginal infection, it is doing so without significant changes in cell numbers or phenotype. Furthermore, there was no evidence for systemic cell infiltration in animals with secondary infections based on the lack of increases in GK 1.5^+ $CD4^+$ cells as detected by flow cytometry or assessed by amplification products for CD4 with regular *Taq* DNA polymerase.

On balance, our results show that during both primary and secondary *C. albicans* vaginal infection there is a lack of changes in resident vaginal T cells and no evidence for systemic $CD4^+$-T-cell infiltration. The lack of local T-cell changes does not imply that vaginal T cells cannot increase in number in response to stimuli. Indeed, vaginal T cells increase in number on in vivo pan-T-cell antibody treatment (19, 38). Additionally, there is evidence that γδ T cells may be important in host defense against vaginitis, since depletion of such cells was reported to increase susceptibility to infection (22). In light of this, we are currently examining activation markers (e.g., CD69 and CD25) and DNA staining as well as cytokines/chemokines to identify any direct evidence for local T-cell activation. Alternatively, antibody-mediated immunity may be responsible for the seemingly acquired *Candida*-specific host response. There is support for this hypothesis in the experimental rat model of *C. albicans* vaginitis (4, 6, 37) but little support clinically (39).

The lack of evidence for systemic T-cell trafficking into the vaginal mucosa during primary and secondary vaginal infections is entirely consistent with our previous immunological observations in the experimental vaginitis model and with conclusions from our clinical studies that showed no involvement of systemic CMI (10, 11, 14, 15). However, this is in contrast with that observed in other experimental models of genital tract infections. In both experimental *Chlamydia trachomatis* and herpes simplex virus type 2 genital tract infections, $CD4^+$ T cells infiltrate to the site of infection (33–35). In each case, this has been shown by either amplification of CD4 mRNA (not detected in naive mice) (35) or staining with GK 1.5 anti-CD4 antibodies (23, 33, 34). The presence of GK 1.5^+ $CD4^+$ cells in the vagina in response to these other genital tract infections also reduces the possibility for an alternative explanation of our results, namely, that the vaginal environment (i.e., the pH) had affected the infiltrating $CD4^+$ cells such that they could no longer be detected by GK 1.5 anti-CD4 antibodies or CD4 primer sets. Thus, it would appear, in contrast to other vaginal infections, that specific adhesion molecules on the vaginal tissue endothelium are not upregulated in response to a vaginal *C. albicans* infection or that the *Candida*-specific Th1-type $CD4^+$ cells in the draining lymph nodes of infected mice do not express the adhesion molecules required to enter the vaginal tissue.

Effect of PMN on *C. albicans* vaginal infection. A second objective of the study was to assess the role of PMN during a vaginal *C. albicans* infection. This is an important issue because PMN are often observed in vaginal lavage fluid of infected animals and systemically-derived PMN have considerable anti-*Candida* activity in vitro (3, 8). However, the presence of PMN is erratic during an experimental vaginal infection and rarely correlates with a reduced vaginal fungal burden. Furthermore, PMN are not normally observed in vaginal smears (KOH) from women with vaginitis (43a). Recently, Black et al. reported a lack of effect of PMN depletion on vaginitis in the presence of estrogen (2). Their interpretation was that PMN may be important in anti-*Candida* vaginal host defense but that estrogen inhibits the ability of PMN to be deployed into the lumen in large enough numbers to affect *C. albicans*. To test this hypothesis, antibodies to PMN (anti-Ly-6G antibodies; PharMingen) (100 μg) or isotype control antibodies (rat immunoglobulin G; Zymed Laboratories, San Francisco, Calif.) were injected intraperitoneally (2) 1 day before and 3 days after vaginal inoculation in the presence or absence of pseudoestrus. The vaginal fungal burden in such animals, measured 5 days postinoculation, showed in two experiments that depletion or reduction of PMN (<1% in spleen preparations compared to 8 to 10% in isotype control antibody-treated mice, as detected by Wright's stain) had no effect on the vaginal fungal burden in the presence ($1.7 \times 10^4 \pm 6.1 \times 10^3$ CFU for anti-PMN-treated mice and $6.4 \times 10^4 \pm 2.3 \times 10^4$ CFU for isotype control antibody-treated mice) or absence ($2.1 \times 10^3 \pm 1.7 \times 10^3$ CFU for anti-PMN-treated mice and $4.4 \times 10^3 \pm 4.6 \times 10^3$ CFU for isotype antibody-treated mice) of pseudoestrus. Thus, despite their presence, PMN do not appear to play a role in host defense against vaginitis, irrespective of the state of estrus. In light of these results, we postulate that the erratic presence of PMN during an infection in the presence or absence of pseudoestrus is due to the normal deployment of PMN during the 2-day diestrus phase of the mouse 4-day menstrual cycle rather than in response to *C. albicans*. If so, it would not appear that a state of pseudoestrus inhibits this

process. Recently, it was reported that the chemokine macrophage inflammatory protein 2 (MIP-2) is involved in the recruitment of PMN during diestrus (44). In support of this, we have observed a similar erratic presence of PMN in lavage fluid of estrogen-treated uninfected mice together with the presence of MIP-2 in the vaginal tissue (unpublished observations).

The lack of effects of PMN against vaginitis is consistent with clinical observations that candidal vaginitis occurs only rarely in neutropenic women (43a). Nevertheless, it is interesting that the vaginal presence of PMN in infected animals does not affect the vaginal fungal burden when PMN kill *C. albicans* readily in vitro (7, 29). Perhaps some form of immunoregulation is present in the vagina that affects the function of PMN against *C. albicans* at that site. More in-depth studies on the vaginal immune response to *C. albicans* will undoubtably shed considerable light on these issues.

This work was supported by Public Health Service grant AI-32556 from the National Institute of Allergy and Infectious Diseases.

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Editor: T. R. Kozel