Estrogen-dependent induction of persistent vaginal candidosis in naïve mice

Östrogen-abhängige Induktion der persistierenden Vaginalcandidose in naiven Mäusen

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Summary
The capacity of estrogen to induce vaginal candidosis (VC) in the absence of previous or concurrent Candida albicans infections was examined. Adult female Balb/c mice were evaluated for vaginal C. albicans burden, C. albicans-specific delayed type hypersensitivity (DTH) responses and vaginal lymphocyte population kinetics at several time-points while receiving weekly injections of 0.5 mg estrogen. Estrogen treatment resulted in the appearance of significant levels of C. albicans vaginal colonization, which persisted for a period of 4 weeks. A marked suppression of DTH responses mounted against subsequent C. albicans challenge was observed. Absolute number of vaginal T lymphocytes gradually increased by several folds especially at weeks 5–6 following the start of estrogen treatment. These results clearly indicate that estrogen, independent of other predisposing factors, is capable of perturbing the commensal relationship between the host and the fungus, which results in the induction of persistent VC.

Zusammenfassung
In der Studie wurde im Tiermodell die Fähigkeit von Östrogen untersucht, die Vaginalcandidose zu induzieren. Adulte weibliche Balb/c-Mäuse wurden konditioniert durch wöchentliche Injektionen von 0.5 mg Östrogen, was vaginale Candidose unabhängig von exogener Exposition über einen Zeitraum von vier Wochen verursachte. An den Versuchstieren wurde die vaginale Candida albicans-Besiedlung, die C. albicans-spezifische DTH-Antwort und die Kinistik der vaginalen Lymphozytenpopulation erfasst. Die Absolutzahlen vaginaler T-Lymphozyten stieg um das Mehrfache bis gegen Ende der Beobachtungszeit an. Die Ergebnisse belegen, dass Östrogen unabhängig von anderen prädisponierenden Faktoren in der Lage ist, die kommensalen Beziehungen zwischen Wirt und Pilz zu stören, was zur persistenten vaginalen Candidose führt.

Key words: Candida albicans, vaginal candidosis, estrogen, delayed type hypersensitivity response, T lymphocytes.

Introduction

Vaginal candidosis (VC) and recurrent vaginal candidosis (RVC) represent a major health problem to women of child-bearing age worldwide.1-4 The leading cause of VC, Candida albicans, asymptptomatically resides as a commensal in the female reproductive tract.5 VC and RVC has been attributed to several predisposing factors including overuse of antimycotic drugs, elevated levels of estrogen in the reproductive tract and compromised host immunity.6 Estrogen has been shown to promote C. albicans adhesion by inducing hyphal growth.7,8 Additionally, estriols, pregnanediols, pregnanetrials and estradiols have been shown to increase germination of C. albicans.9,10 The pleotropic effects of estrogen on the epithelium of the reproductive tract are thought to facilitate and enhance C. albicans adhesion, growth and colonization.5,11,12 Several investigators have used estrogen in conjunction with C. albicans vaginal inoculation to construct animal models of persistent VC; among these models is the estrogen-dependent VC (EDVC) murine model.13-15 This model has proved to be useful in evaluating the various aspects of pathogenesis of VC as VC in the murine system is transient and only lasts for few days.14 Among the several important findings obtained using this model was the observation that estrogen treatment results in persistent VC that it results in suppressed delayed type hypersensitivity (DTH) responses13 and that it leads to a significant increase in vaginal T lymphocyte numbers especially the numbers of CD8+ T cells.16,17 These findings strongly point to the possibility that VC and RVC can be directly correlated with the presence of elevated levels of estrogen in the reproductive tract; this may apply irrespective of the immune status of the host or the contribution of other possible predisposing factors. This is further supported by the observation that a significant percentage of RVC cases occur in women with competent immunity.4,18

To further investigate the role of estrogen in the pathogenesis of VC independent of other predisposing factors, adult naïve non-germ free mice were treated with estrogen on weekly basis. Mice were tested for vaginal C. albicans burden and for C. albicans-specific DTH responses at different time-points while receiving estrogen. Additionally, at different time-points following the start of estrogen treatment, vaginal T lymphocytes were isolated, enumerated and examined for the expression of CD3, CD4 and CD8 T-cell surface markers. Absolute number of the various vaginal T-cell subsets were also calculated as mean values of evaluating T lymphocyte population kinetics in this model.

Materials and methods

Mice

Non-germ free female (12–14 week old) Balb/c mice raised at the Hashemite University vivarium were used throughout the study. Mice were treated with estrogen on weekly basis by subcutaneous administration of 0.5 mg estradiol valerate dissolved in 0.1 ml sesame oil (Schering, Berlin, Germany).

Evaluation of C. albicans colonization

Mice were killed at days 1, 2, 3, 5, 8, 10, 16, 23, 31, 38, 52, and 62 following the start of estrogen treatment; vaginal tissues were isolated from two mice per group, examined for the presence of white lesions characteristic of candidal infection. Tissues were trimmed and homogenized in 10 ml sterile physiologic saline (SPS) using a sterile glass homogenizer (Ystral GmbH, Göttingen, Germany); serial 10-fold dilutions were prepared from each homogenate. Aliquots (1 ml) of the various dilutions were poured into culture plates containing 10 ml Sabouraud glucose Agar (SDA; Difco, Detroit, MI, USA) with chloramphenicol at 50 mg l-1. Plates were left to dry and then incubated at 37 °C; each sample dilution was cultured in triplicate. Yeast colonies were counted 48 h after plating and colonization results were expressed as mean colony-forming units (CFUs) per vagina. Confirmation of C. albicans colonization was carried out by subculturing growing colonies on CHROMagar color-based selective media (a kind gift from CHROMagar, Paris, France).

Examination of DTH responses

Candida albicans-specific DTH responses were measured as previously described.19, 20 Briefly, mice were right footpad-challenged with 2 × 10^7 heat-killed C. albicans (ATCC strain 36082; a kind gift from Dr M. Ghannoum, Mycology Reference Laboratory, University Hospital of Cleveland, Cleveland, OH, USA) in 50 µl pyrogen-free saline at 2, 3, 4, 5, and 6 weeks following the start of estrogen treatment. The left footpad of all mice received 50 µl of SPS as a control. Footpad swelling was measured 48 h later using a Schnelltaster caliper (H. T. Kroplin Hessen, Schüchtern, Germany). The reaction was considered as positive when the difference in swelling between the right and left footpads was >0.2 mm.21
Isolation and phenotypic profiling of vaginal T lymphocytes

Vaginal T lymphocytes were isolated as previously described. Briefly, vaginas of live to six mice per group per time-point were isolated, flushed with normal saline, opened up longitudinally, cut into 2 mm pieces and pooled. Tissue pieces were placed in 50 ml warm RPMI-1640 (Sigma Chemicals, St Louis, MO, USA) solution containing 10 mmol l\(^{-1}\) EDTA and 10 mmol l\(^{-1}\) dithiothreitol (DTT), tissue pieces were stirred for 30 min at 37 °C. The suspension was filtered through 1 g nylon wool column moisturized with warm Hank’s balanced salt solution (HBSS) (Sigma), the filtrate was centrifuged and cell pellet was suspended in 1 ml HBSS. Cells were counted and prepared for staining: 10^4–10^5 cells in 100 μl HBSS were used per sample for flow cytometric analysis. Biotin-labeled anti-CD3 (KT3), phycoerythin (PE)-labeled anti-CD4 (YTS191.1) and fluorescein isothiocyanate (FITC)-labeled anti-CD8 (KT15) antibodies (Serotec Ltd, Oxford, UK) were used for T cell phenotypic characterization. For single-color analysis, biotin-labeled anti-CD3 was added at 1 μl per sample, left to react for 30 min on ice, cells were then centrifuged, washed with 100 μl HBSS, cell pellet was then resuspended in 100 μl HBSS and reacted with PE-labeled streptavidin. For dual-color analysis, each cell sample was reacted with 2 μl FITC-labeled anti-CD8 and 1 μl PE-labeled anti-CD4, for 30 min on ice prior to washing. Bimapping of vaginal lymphocytes was based on cell size and granularity of control lymphocyte populations (splenic and lymph node cells). Flow cytometric analysis was performed on a Becton Dickinson flow cytometer (Becton Dickinson, Mountain View, CA, USA); 10^4 cells were analyzed per sample and data were collected and analyzed using an Apple Macintosh computer equipped with Simulset software (Becton Dickinson).

Statistical analysis

ANOVA test was employed to determine levels of significance within the experimental group (one-way ANOVA) and between the experimental and control groups (two-way ANOVA) regarding DTH responses and lymphocyte absolute numbers. Statistical analysis of T lymphocyte subpopulations was conducted using the Student’s t-test (significance level at \(P < 0.05\)).

Results

Levels of vaginal C. albicans colonization were evaluated at days 1, 2, 3, 5, 8, 10, 16, 23, 31, 38, 52, and 62 following the start of estrogen treatment. Mice used were naïve mice that tested negative for C. albicans prior to treatment as evidenced by absence of C. albicans growth in vaginal swab cultures (data not shown). To ensure absence of inadvertent contamination by C. albicans, vaginas were taken from treated mice as well as control counterparts at every time-point of the study period. Significant levels of vaginal fungal burden were detected in homogenate cultures from treated mice (Fig. 1), which gradually increased to peak at around day 5 following the start of estrogen treatment. The infection lasted for a period of 30–32 days. Except for the gastrointestinal tract which showed minimal levels of C. albicans colonization, no detectable levels of C. albicans were observed in the liver, spleen or lymph nodes isolated from treated or control mice (data not shown). A significant percentage (21.6%) of mice died

Figure 1 Candida albicans vaginal fungal burden in estrogen-treated mice. Adult naïve female Balb/c mice receiving weekly injection of 0.5 mg estrogen were tested for the presence of C. albicans at days 1, 2, 3, 5, 8, 16, 23, 31, 38, 52, and 62 following the start of the estrogen treatment. Mean cfu counts/vagina ± SD as presented here were calculated based on two separate experiments.
mostly as a result of estrogen-induced VC; mortality within the treated group occurred mostly between weeks 4 and 5 following the start of the experiment (Table 1). All mice included in the control group survived throughout the study period. These results suggest that estrogen alone is capable of inducing pathogenic episodes of VC independent of exogenous exposure to the fungus.

*Candida albicans*-specific DTH responses were evaluated in estrogen-treated mice at 2, 3, 4, 5, and 6 weeks post the start of treatment. Treated mice exhibited detectable *C. albicans*-specific DTH responses (3.6 mm) as early as week 2 post-treatment compared with the control group (*P* < 0.005), which slowly but significantly dropped to background levels by week 6 post-treatment (*P* < 0.0305 for week 2 vs. week 6) (Fig. 2).

DTH responses in mice vaginally inoculated with *C. albicans* in the presence of estrogen were shown to be only slightly higher (3.7–3.8 mm) than those observed in treated uninfected mice. However, DTH responses observed in mice vaginally inoculated with *C. albicans* in the absence of estrogen were shown to be significantly greater (=4.4 mm) than those being reported here. These results suggest that, although estrogen-induced VC is capable of conferring systemic protection against subsequent *C. albicans* infections, it seems to do so at a subdued level when compared with VC resulting from *C. albicans* infection in the absence of estrogen.

Vaginal lymphocytes isolated from estrogen-treated mice were evaluated in terms of their phenotypic profile and absolute numbers at different time-points following the start of the experiment. Vaginal lymphocytes were also isolated from control mice and evaluated for comparative analysis. It was difficult to discern a reliable pattern of vaginal T lymphocyte population kinetics based on flow cytometric-generated cell percentage data alone as the total number of vaginal T lymphocytes and that of the various T-cell subsets increased in a very significant and possibly disproportionate fashion. Therefore, absolute numbers of CD3⁺, CD8⁺, and CD4⁺ vaginal T cells were calculated based on cell subset percentage and total T-cell numbers. As shown in Table 2, a gradual increase in all T-cell subsets occurred during the course of infection, the increase was most pronounced toward the end of infection and during the period of infection resolution. At day 38, the total number of T cells was about 17-folds greater than that observed in control mice; for CD8⁺ and CD4⁺ vaginal T-cell subsets the increase was 23- and sixfolds respectively. Within the treated group, the increase was gradual but significant (*P* < 2.39 × 10⁻⁶ for day 38 vs. day 1, *P* < 1.83 × 10⁻⁶ for day 38 vs. day 16 and *P* < 1.96 × 10⁻⁶ for day 38 vs. day 23). It is clear that the greater majority of CD3⁺ T cells were of the CD8⁺ phenotype suggesting that this cell subset in particular is of great importance in protection.

![Figure 2](image)

**Figure 2** Estrogen-treatment dependent vaginal candidosis results in the induction of delayed type hypersensitivity (DTH). DTH responses were evaluated by measuring footpad swelling 48 h after right footpad challenge of naïve mice (control) and estrogen-treated mice with 2 × 10⁷ heat-killed *Candida albicans* cells. Data presented here represent the average ± SD of two separate experiments.

### Table 1 Mortality rates in mice with estrogen-induced vaginal candidosis.

<table>
<thead>
<tr>
<th>Day</th>
<th>Number of animals</th>
<th>Number of dead animals</th>
<th>Percentage mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>60</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>23</td>
<td>30</td>
<td>1</td>
<td>3.3</td>
</tr>
<tr>
<td>26</td>
<td>29</td>
<td>2</td>
<td>6.9</td>
</tr>
<tr>
<td>29</td>
<td>27</td>
<td>2</td>
<td>7.4</td>
</tr>
<tr>
<td>30</td>
<td>25</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>31</td>
<td>18</td>
<td>3</td>
<td>16.7</td>
</tr>
<tr>
<td>38</td>
<td>9</td>
<td>4</td>
<td>44.4</td>
</tr>
<tr>
<td>Total mortality</td>
<td>13</td>
<td></td>
<td>21.6</td>
</tr>
</tbody>
</table>

1. Number of animals at any specific time-point was calculated as the original (60 mice) – dead and killed animals at each time-point. Data shown is the average of two separate experiments.

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Estrogen-induced vaginal candidosis

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against vaginal *C. albicans* infection; the increase in CD8+ T cells in the treated group was very significant compared with the untreated group (*P* < 0.001). The significance of CD4+ T cells, which have also shown a significant increase during the infection period (*P* < 0.05), must not be discounted.

**Discussion**

Several important points pertinent to estrogen as a major predisposing factor in the pathogenesis of VC can be concluded from the results of this study. The capacity of estrogen to induce VC independent of exposure to or infection by *C. albicans* was clearly demonstrated by the positive vaginal homogenate culture results and CFU counts. It must be noted that whereas persistence of vaginal *C. albicans* infection in this mouse model lasted for about 4 weeks, persistence of vaginal *C. albicans* infection in the EDVC murine model was reported to last for more than 8 weeks.14 Additionally, vaginal *C. albicans* burden was lower in this mouse model compared with that reported in the EDVC model.15 Nonetheless, the evident capacity of estrogen to induce significant levels of VC independent of other factors is indicative of the prominent role estrogen plays in the pathogenesis of VC. The capacity of estrogen to induce VC was also confirmed by the observation that findings usually associated with the disease in the conventional EDVC mouse model15,26 were also observed in the current model. First, a significant percentage of estrogen-treated mice died as a result of *C. albicans* infection. Secondly, treated mice exhibited suppressed DTH responses compared with *C. albicans* vaginally inoculated untreated mice. Thirdly, a significant increase in vaginal T-lymphocyte numbers was consistently noted.

Although the mechanism by which estrogen induces VC independent of direct infection by the pathogen cannot be readily explained, it is likely that estrogen, via various converging effects, perturbs the commensalistic balance between the fungus and the host. This may occur by a combination of estrogen-dependent changes that ultimately result in converting *C. albicans* from a commensal residing within the reproductive tract environment into a pathogen colonizing and harming the host. Estrogen was reported to induce changes in the vaginal mucosa that facilitate *C. albicans* adhesion, growth and colonization.8,27 It was also shown to act on the fungus itself leading to germ tube formation,10 which is well-known to increase the pathogenic potential of the fungus.7–10 Estrogen was reported to suppress the cellular immunity of the host28,29 further contributing to the creation of a more favorable environment for the pathogenic potential of the fungus to be expressed. Consistent with this line of reasoning, suppression of DTH responses suggests that estrogen compromises host immunity. This, in turn, may help explain the recurrence of VC in young healthy women.1,2,6,30 Interestingly, findings similar to those reported here, especially those pertaining to the induction of VC and the development of a state of compromised immunity have also been observed in women receiving hormonal therapy.6,30 In conclusion, elevated levels of estrogen in the reproductive tract seems to perturb the commensal asymptomatic relationship between the host and the fungus, which results in pathogenic episodes of VC independent of exposure to exogenous *C. albicans* infection.

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### Table 2

Mean absolute number and percentage of total, CD3+, CD8+, CD4+ and CD4+ CD8+ double positive (DP) vaginal lymphocytes isolated from naïve untreated and estrogen-treated mice

<table>
<thead>
<tr>
<th>Days post start of treatment</th>
<th>Number of vaginal T cells per vagina × 10^3</th>
<th>CD3+</th>
<th>CD8+</th>
<th>CD4+</th>
<th>DP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Number</td>
<td>%</td>
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<td>%</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0‡</td>
<td>30</td>
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<td>97</td>
<td>12</td>
<td>40</td>
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<tr>
<td>8</td>
<td>108</td>
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<td>22.2</td>
<td>1.4</td>
<td>1.3</td>
</tr>
<tr>
<td>16</td>
<td>89</td>
<td>26</td>
<td>30.2</td>
<td>7.5</td>
<td>8.4</td>
</tr>
<tr>
<td>23</td>
<td>94</td>
<td>74</td>
<td>22.2</td>
<td>21.5</td>
<td>9.2</td>
</tr>
<tr>
<td>31</td>
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<td>464</td>
<td>96.1</td>
<td>163</td>
<td>33.7</td>
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<tr>
<td>38</td>
<td>520</td>
<td>509</td>
<td>97.9</td>
<td>280</td>
<td>53.8</td>
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<tr>
<td>52</td>
<td>387</td>
<td>333</td>
<td>86.0</td>
<td>157</td>
<td>40.6</td>
</tr>
</tbody>
</table>

1Cell number; 2control mice; data shown is the average of two separate experiments; standard deviation was <6.8% throughout.
References