Candida-Specific Cell-Mediated Immunity Is Demonstrable in Mice with Experimental Vaginal Candidiasis

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Women with recurrent vulvovaginal candidiasis often demonstrate a down-regulation of cell-mediated immunity (CMI) to Candida albicans detected by a lack of cutaneous delayed-type hypersensitivity (DTH) to Candida antigens. However, the role of systemic CMI as a host defense mechanism against recurrent vulvovaginal candidiasis is not well understood, in part because of the lack of a well-defined murine model of vaginal candidiasis. The present study was undertaken to determine: (i) whether soluble Candida culture filtrate antigens (CaCF) could be used to induce and detect Candida-specific CMI in mice and (ii) whether these antigens would be useful in detecting systemic CMI in mice given an experimental Candida vaginal infection. To this end, mice were immunized subcutaneously with CaCF in complete Freund's adjuvant, and within 7 days they developed Candida-specific DTH reactivity detected by footpad swelling (increase in footpad thickness, 0.36 mm) 24 h after footpad challenge with CaCF. Adoptive transfer studies showed that the DTH responsiveness was elicited by CD4+ DTH T cells. In mice given a vaginal inoculum of C. albicans blastoconidia (5 x 105), footpad challenge with CaCF resulted in positive DTH responses (0.24 mm) as early as 1 week, responses similar to immunization in 2 to 3 weeks (0.33 mm), and sustained low levels of DTH reactivity (0.15 mm) through 10 weeks of vaginal infection. Vaginal lavage cultures revealed that peak vaginal Candida burden occurred 1 week post-vaginal inoculation (102 CFU) and declined 16-fold by week 10. These results provide evidence that Candida-specific systemic CMI is generated and can be detected longitudinally in mice with Candida vaginitis by a multiantigen preparation of Candida organisms which both initiates and detects Candida-specific CMI.

Recurrence vulvovaginal candidiasis (RVVC) is an opportunistic mucosal infection (39). Candida species are the second most common cause of vaginal infections otherwise healthy females (10). An estimated 75% of all women will experience an episode of Candida vaginitis once in their lifetime, with up to 5% experiencing RVVC (20-22). Candida albicans is the causative agent in approximately 85 to 90% of patients with symptomatic yeast vaginitis (15, 20, 28, 31). Antimycotic agents, although highly effective for individual attacks, do not prevent recurrence in women with chronic vulvovaginal candidiasis or RVVC. Unlike infrequent episodes of vaginitis precipitated by pregnancy, oral contraceptives, uncontrolled diabetes mellitus, and particularly the use of antibiotics, there are no recognized exogenous predisposing factors for RVVC (38). RVVC is presumed to result from diminished host defense mechanisms that increase susceptibility to symptomatic infection, including the enhanced conversion of C. albicans from vaginal commensal to pathogen. It has been postulated that RVVC results from a down-regulation of cell-mediated immunity (CMI) similar to the immunoregulatory events associated with chronic mucocutaneous candidiasis (1, 5, 13, 14, 18, 32, 35, 42), which are often mediated by suppressor cells (13, 34, 35).

The finding of reduced systemic CMI to Candida infection in women with RVVC (16, 19, 41, 43, 44) supports the contention that CMI is an important antifungal host defense mechanism at the mucosal level. In addition, the fact that vaginal candidiasis has been reported for 25% of human immunodeficiency virus-infected women (36) emphasizes the importance of CMI in protection against Candida vaginal infection. However, little is known about how CMI functions as a natural mucosal defense mechanism in the vagina and how it is regulated.

Progress in understanding the role of CMI in vaginal candidiasis has been hampered by the lack of a well-defined animal model to study Candida-specific CMI during experimental C. albicans vaginal infections. Although an estrogen-dependent murine model of experimental Candida vaginitis has been available for several years (37, 40), to date it has been used primarily to test the efficacy of antifungal agents and to study adherence of Candida species to epithelial cells. There is no information on the role of humoral or cell-mediated immune mechanisms associated with experimental Candida vaginal infection or on whether immune responsiveness is demonstrable in vivo or in vitro. In contrast, the importance of CMI in gastrointestinal host resistance to C. albicans has been demonstrated in both humans (4, 11, 25) and animal models (2, 6-8, 12, 30). The use of murine models has allowed the identification of both lymphocyte populations that protect against Candida gastrointestinal infection (7, 8, 12) and those that down-regulate Candida-specific CMI, thus creating susceptibility to infection (3, 17). Additionally, athymic (2, 6) and severe combined immunodeficient (T- and B-cell-deficient) (30) mice have been particularly useful for studying susceptibility to gastrointestinal candidiasis.

The present study was undertaken to determine whether Candida-specific CMI, detected by delayed-type hypersensitivity (DTH), could be demonstrated in vaginally infected mice with a complex culture filtrate antigen of C. albicans.

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MATERIALS AND METHODS

Mice. Female CBA/J (H-2^k) mice, 8 to 10 weeks of age, purchased from the Jackson Laboratory (Bar Harbor, Maine) were used throughout these studies.

Antigens. *C. albicans* culture filtrate antigens (CaCF) were prepared from *C. albicans* 3153A serotype A (3153A) (American Type Culture Collection, Rockville, Md.) according to the methods described for the production of *Cryptococcus neoformans* culture filtrate antigens (9). Briefly, the growth medium for *C. albicans* (50% Phtyone-peptone) was prepared and dialyzed against H_{2}O (12,000- to 14,000-molecular-weight exclusion) (Spectrum Industries, Houston, Tex.) for 5 h at 7°C. After overnight incubation at 4°C the dialysis tubing was removed, the dialysate was autoclaved, and sterile glucose (1%) was added. The dialysate medium was inoculated with a fresh blastoconidium broth culture of *C. albicans* 3153A (2 x 10^7/ml) and incubated in a shaker H_{2}O bath at 25°C for 3 days. The supernatants from the culture were concentrated 10-fold from a 10,000-molecular-weight exclusion membrane (Amicon Corp., Danvers, Mass.) during washing with 2 to 3 volumes of phosphate-buffered saline (PBS). The final preparation was passed through a sterile filter (Nalgene Co., Rochester, N.Y.) and stored at -70°C.

Protein concentrations for CaCF were between 0.20 and 0.50 mg/ml, as measured by the Lowry trichloroacetic acid precipitation kit (Sigma Chemical Co., St. Louis, Mo.). Heat-killed blastoconidia (HKB) were prepared by incubating a fresh 3153A blastoconidium broth culture at 60°C for 2 h. Mannan (MAN) was prepared as previously described (33). Other antigens used in murine assays included *C. neoformans* culture filtrate antigen (CneF) kindly provided by Juneann Murphy (University of Oklahoma Health Sciences Center, Oklahoma City). To ensure that the lipopolysaccharide (LPS) concentration was negligible in the culture filtrate antigens, the *L. monocytogenes* amebocyte lystate test (Sigma) was performed. Results showed that CaCF had 0.06 endotoxin units of LPS or less per ml, which is considered negligible.

Immunization and detection of DTH reactivity. Mice were immunized with CaCF emulsified 1:1 in complete Freund’s adjuvant (CFA). The mice were injected subcutaneously with 20 to 50 μg of CaCF in CFA (CaCF-CFA) at two sites at the base of the tail (0.1 ml per site). Control mice were injected with PBS-CFA. Mice were footpad challenged 6 days after immunization by injecting one hind footpad with 50 μl (10 μg) of CaCF and the contralateral footpad with 50 μl of PBS. Footpad thickness was premeasured with an Interapid micrometer (Borrel and Dunning Inc., Novi, Mich.). Footpad thickness was measured again to determine DTH swelling reactivity 18 to 24 h after challenge. For specificity studies, animals injected with several different antigens were used for immunization and footpad challenge. These included animals immunized with culture filtrate medium (CFmed)-CFA or CneF-CFA (29) and challenged 6 days later with CaCF and also CaCF-CFA-immunized mice challenged 6 days later with CneF or CFmed. Tests were also performed for *Candida*-related antigen specificity in which CaCF-CFA-immunized mice or HKB-immunized mice (5 x 10^7 HKB in 0.5 ml of PBS given intraperitoneally) were challenged with CaCF or MAN.

Phenotype of DTH-producing cells. To analyze the cell population responsible for DTH reactivity, 0.5 mg of either anti-L-JT4 (anti-CD4, GK 1.5; American Type Culture Collection) or anti-L-JT2 (anti-CD8, HB129; American Type Culture Collection) antibodies were administered to mice intravenously in 0.5 ml of PBS on days -1 and 3. Mice were immunized with CaCF-CFA on day 0. DTH reactivity was measured on day 7. To confirm the action of the antibodies in vivo, spleen cells from treated animals were labeled with fluorescein isothiocyanate-conjugated anti-CD4 or anti-CD8 antibodies and analyzed with a fluorescence-activated cell sorter. Results showed that compared with the numbers of CD4 and CD8 cells in spleens of PBS-treated mice, the number of CD4 cells in spleens of anti-CD4-treated mice was reduced by 70 to 75%, whereas the number of CD8 cells remained unchanged or was slightly augmented by the lack of CD4 cells. Similar results were obtained with anti-CD8-treated mice: the number of CD8 cells in the spleens was reduced by 68 to 70%, while the number of CD4 cells remained unchanged.

Demonstration of experimental murine *Candida* vaginal infection. Vaginitis in rodents is inducible only under conditions of pseudoeoestrus (37, 40). Accordingly, 72 h prior to vaginal inoculation, mice were treated subcutaneously with 0.5 mg of estradiol valerate (E. R. Squibb & Sons, Inc., Princeton, N.J.) dissolved in sesame oil. Estrogen administration continued weekly until completion of the study. *C. albicans* 3153A blastoconidia (5 x 10^8) obtained from a fresh stationary-phase culture were inoculated in solution with 20 μl of PBS into the vaginas of estrogen-treated mice. Control animals received 20 μl of PBS. At weekly intervals beginning 1 week after vaginal inoculation and continuing for 10 weeks, groups of four mice were footpad challenged with 10 μg (50 μl) of CaCF, and 24 h later the footpad swelling was measured and the mice were sacrificed. Vaginal lavage with 100 μl of PBS was performed on mice immediately after sacrifice. Vaginal tissue was gently scraped as the fluid was collected. Serial 1:10 dilutions of the lavage fluid were made, and 50 μl of each dilution was plated onto Sabouraud dextrose agar plates and incubated for 48 to 72 h at 30°C. To verify the presence of *C. albicans* on the plates after 48 to 72 h, randomly selected colonies were inoculated into 0.5 ml of fetal calf serum (GIBCO), incubated for 2 h at 37°C in 10% CO_2, and analyzed for the presence of germ tubes microscopically. The presence of germ tubes was considered confirmation of *C. albicans*. Vaginal lavage fluid was also analyzed microscopically for the presence of hyphae by wet-mount slide preparation, and vaginas from randomly selected mice were dissected, fixed in formalin, and embedded in paraffin. Paraffin sections were prepared and stained with hematoxylin for histological examination.

Statistical analysis. The unpaired Student's t test was used to analyze the data. Significant differences were defined as a confidence level at which P was <0.05.

RESULTS

CaCF induces DTH in mice. To determine whether CaCF could induce DTH in mice, CBA/J (H-2^k) mice were injected subcutaneously with 20 to 50 μg of CaCF-CFA. On designated days after immunization, the mice were footpad challenged with 10 μg of CaCF, and the swelling of footpads was measured over a 48-h period with a micrometer. The results in Fig. 1A show that when swelling responses were recorded 24 h after footpad challenge, DTH reactivity was demonstrable as early as 4 days after immunization (mean ± standard error of the mean [SEM], 0.16 ± 0.02 mm increase in footpad thickness) compared with the results for control mice challenged with CaCF. The DTH reactivity peaked at day 7 (0.48 ± 0.09 mm) and then declined by days 11 and 13 (0.22 ± 0.03 mm). In this study, DTH did not differ whether the contralateral footpad received PBS (used to wash the antigen) or

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When CaCF-CFA-immune mice were challenged with CaCF, significant footpad swelling responses (0.07-mm) and positive (0.46-mm) DTH reactivity were observed. With either CaCF (P < 0.025 compared with the value for group 1) or MAN (P < 0.001 compared with the value for group 3). Alternatively, CneF-CFA-immunized mice, which showed DTH reactivity following challenge with CneF (P < 0.05 compared with the value for nonimmunized CneF-challenged mice), could not mount a swelling response to CaCF. Similarly, Cmef-CFA-immunized mice failed to show swelling after footpad challenge with CaCF.

Phenotype of cells responsible for DTH. To determine the phenotype of the cells responsible for DTH reactivity, mice were depleted in vivo of CD4 or CD8 T cells and immunized with CaCF-CFA. The DTH reactivities measured on day 7 are shown in Fig. 3. DTH reactivity was not affected in mice given PBS as a control. Mice treated with anti-L3T4 antibodies showed a significant reduction in DTH reactivity (P < 0.0005) compared with positive controls, whereas mice treated with anti-Lyt 2 antibodies demonstrated normal DTH reactivity.

CMI reactivity during experimental Candida vaginitis infection. In order to (i) provide evidence that vaginal infections could be established in CBA/J mice and (ii) determine whether CaCF could be used to monitor CMI generated as a result of vaginal infection, mice were given a vaginal inoculum of 5 × 10^6 viable C. albicans blastoconidia, and CaCF-mediated DTH reactivity and vaginal Candida burden were

FIG. 1. Kinetic study of DTH reactivity induced by CaCF. Groups of four mice were immunized with CaCF-CFA and footpad challenged with CaCF. Control animals received PBS-CFA. (A) Footpad swelling responses (reported as increases in thickness, in millimeters) 24 h after challenge on various days after CaCF-CFA immunization; (B) footpad swelling responses at various times after footpad challenge in mice immunized with CaCF-CFA and challenged 6 days later. Data are from a representative experiment, performed in duplicate. Error bars show SEM.

CFMed prepared similarly to the antigen (data not shown). DTH reactivity was not demonstrable in mice injected with PBS-CFA and footpad challenged with CaCF. Figure 1B shows the kinetics of footpad swelling after challenge of 6-day immunized mice. Significant footpad swelling in immunized mice declined from 0.45 ± 0.02 mm at 4 h to 0.38 ± 0.03 mm by 8 h and then increased to the maximum swelling (0.48 ± 0.06 mm) at 24 h. Negligible DTH reactivity was detected 48 h after challenge in immunized mice. Footpad swelling in mice injected with PBS-CFA and challenged with CaCF steadily declined to near the baseline by 24 h and remained at the baseline throughout the 48-h period.

CaCF induces antigen-specific DTH reactivity. To determine whether the DTH reactivity induced by CaCF represented Candida-specific responses, CaCF-CFA- and HKB-immunized mice were tested for DTH reactivity to CaCF, Candida-related cell wall antigens, and an irrelevant fungal antigen. The results are illustrated in Fig. 2. Groups 1 and 2 represent the unimmunized (negative) and CaCF-CFA-immunized (positive) DTH swelling responses after CaCF footpad challenge, respectively. When CaCF-CFA-immunized animals were footpad challenged with MAN, a significant swelling response occurred (P < 0.0025 compared with the value for nonimmunized MAN-challenged mice [group 3]). When CaCF-CFA-immune mice were footpad challenged with CneF, insignificant swelling was observed. In additional specificity experiments, significant DTH reactivity was observed in HKB-immune mice footpad challenged with either CaCF (P < 0.001) or MAN (P < 0.0025) compared with the value for nonimmunized CaCF-challenged mice.

FIG. 2. Specificity of CaCF-mediated DTH. Groups 1 and 2 represent the negative (0.07-mm) and positive (0.46-mm) DTH swelling responses for CaCF footpad challenge without and with CaCF immunization, respectively. Each group contained five mice. Data are from one representative experiment, performed in duplicate. Ag, antigen; NS, not significant.

FIG. 3. Phenotype of DTH-producing cells. Groups 1 and 2 are positive and negative controls, respectively, for DTH reactivity. Each group contained four mice. Antibodies (0.5 mg per mouse) and PBS were administered intravenously. Data are from one representative experiment, performed in duplicate. NS, not significant.

<table>
<thead>
<tr>
<th>Group</th>
<th>In vivo treatment day 0</th>
<th>Immunize CaCF-CFA day 7</th>
<th>Mean increase in footpad thickness [mm] ± SEM</th>
<th>P to group 2</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>−</td>
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<td>0.10 ± 0.02</td>
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<tr>
<td>2</td>
<td>−</td>
<td>+</td>
<td>0.38 ± 0.06</td>
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<tr>
<td>3</td>
<td>PBS</td>
<td>+</td>
<td>0.50 ± 0.05</td>
<td>NS</td>
</tr>
<tr>
<td>4</td>
<td>anti-L3T4</td>
<td>+</td>
<td>0.30 ± 0.04</td>
<td>NS</td>
</tr>
<tr>
<td>5</td>
<td>anti-Lyt 2.1</td>
<td>+</td>
<td>0.40 ± 0.04</td>
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monitored longitudinally for 10 weeks. The mean results from three infection studies are shown in Fig. 4. In these studies, estradiol-treated mice given a vaginal inoculum of \textit{C. albicans} developed DTH reactivity within 1 week (increase in footpad thickness, 0.25 ± 0.015 mm). DTH reactivity peaked at 2 weeks (0.33 ± 0.055 mm) and was still at significant levels at 4 weeks (0.13 ± 0.02 mm). Reactivity declined to negligible levels by 6 weeks and remained at low levels (0.10 to 0.12 mm) through 10 weeks (Fig. 4A). Numbers of \textit{C. albicans} blastoconidia quantitated from vaginal lavage cultures were at highest levels (10^2 ± 10^6 CFU/100 μl of lavage fluid) through 1 week post-vaginal inoculation. By week 4 of infection, \textit{C. albicans} levels declined eightfold to 1.2 × 10^4 CFU ± 1.5 × 10^4 CFU/100 μl of lavage fluid. Levels of \textit{C. albicans} remained at approximately 10^4 CFU through week 8 before declining to an average of 6 × 10^3 CFU by week 10 (Fig. 4B). This represented a 16-fold decrease in vaginal \textit{Candida} burden. Biweekly germ tube tests on randomly selected colonies from the vaginal lavage cultures confirmed that \textit{C. albicans} was isolated from the mouse vaginas. Conversely, estrogen-treated mice inoculated vaginally with only PBS failed to develop DTH activity and the lavage fluid contained no \textit{C. albicans} organisms, evidenced both by the lack of growth on Sabouraud dextrose agar and the lack of yeast elements in wet-mount slide preparations. For additional evidence of infection, histological sections prepared from randomly selected vaginas were stained with hematoxylin. Histological examination showed no evidence of hyphae in mice inoculated with PBS (Fig. 5A), whereas hyphae were readily apparent in mice that

received a vaginal inoculum of \textit{C. albicans} (Fig. 5B).

**DISCUSSION**

Whereas murine models have been useful in studying CMI to gastrointestinal candidiasis (2, 6, 7, 30), to date animal models have not been employed to investigate \textit{Candida}-specific systemic CMI during vaginal candidiasis. The purpose of this study was to determine whether a multiantigen culture filtrate preparation of \textit{C. albicans} could be used to detect and measure, by DTH reactivity, systemic CMI in mice given a vaginal infection with \textit{C. albicans}. We chose a culture filtrate preparation of \textit{Candida} antigens because soluble culture filtrate antigens can be easily used to induce and detect CMI responses and because they consist of several different \textit{Candida} antigens capable of eliciting DTH responsiveness.

Initial studies were designed to assess the efficacy of the \textit{Candida} antigen preparation by determining its ability to induce and detect systemic CMI in naive mice. Results showed that DTH reactivity could be induced and detected
in vivo in mice following immunization with CaCF-CFA. DTH reactivity peaked 7 days after immunization and 24 h after footpad challenge. This pattern is consistent with the classic pattern of DTH responsiveness to Candida antigens (8, 12), as well as with other fungal models of DTH, i.e., C. neoformans (29) and Paracoccidioides brasiliensis (23). The DTH reactivity was Candida-specific by virtue of cross-reactivity with Candida antigens (MAN and HKB) but not with unrelated fungal antigens prepared from C. neoformans. Abrogation of DTH reactivity in mice treated with anti-CD4 antibodies but not anti-CD8 antibodies confirmed that CD4+ DTH T cells were responsible for eliciting DTH reactivity. Taken together, the results suggested that CaCF is capable of inducing classic CMI and would be useful to detect, quantitate, and characterize systemic Candida-specific CMI during experimental Candida vaginitis.

Evidence for experimental Candida vaginitis in rodents during conditions of pseudoestrus (37, 40) comes from superficial association of hyphae with estrogen-mediated proliferation of epithelial layers of the vagina. This is detected by quantitation of C. albicans in vaginal lavages and by the presence of hyphae in both histological preparations of dissected vaginas and wet-mount slide preparations of undiluted lavage fluid. However, no data for CBA/J mice have been collected with respect to kinetics of murine vaginal infections (e.g., whether CMI is generated and can be quantitated during the course of infection) have been collected.

In this study, we confirmed that Candida vaginitis can be achieved in CBA/J (H-2b) mice under conditions of pseudoestrus. This particular strain was chosen because H-2b mice are less resistant to Candida infection than H-2a mice (26), which allows the potential generation of immunity, and not so resistant that infection cannot occur. With this model, we provide evidence that systemic CMI is generated as a result of Candida vaginal infection and can be detected and quantitated longitudinally by DTH reactivity with CaCF as the challenge antigen. This is similar to results obtained with experimental models of gastrointestinal candidiasis in which systemic CMI is demonstrable following introduction of Candida spp. into the gastrointestinal tract (7, 8, 12). The CMI responsiveness during vaginal infection, as detected by DTH reactivity, peaked 2 weeks after vaginal inoculation. This corresponded to 1 week after peak vaginal Candida burden was reached. It is noteworthy that peak levels of DTH reactivity in vaginally infected mice were equivalent to the peak level of DTH reactivity in mice immunized with CaCF-CFA, indicating that Candida antigens originating from the vagina can stimulate DTH reactivity similarly to those administered systemically with adjuvant. In contrast, however, to the short-lived pattern of DTH reactivity in CaCF-CFA-immunized mice (Fig. 1), DTH reactivity in vaginally infected mice was still relatively high through 3 weeks of infection and was sustained at low levels for up to 10 weeks. This suggests that Candida antigens are continually being released at low levels throughout the period of infection, a possibility that is supported by the steady but slow decline in vaginal Candida burden throughout the 10-week period.

Although a steady decline in vaginal Candida burden was evident during the 10-week period, complete clearance of Candida organisms was rarely observed. The lack of clearance of Candida organisms in the presence of demonstrable systemic CMI may in part be due to the dominant pharmacological effect of estrogen, which provides an environment that facilitates the adherence (27) and proliferation (24) of Candida organisms. Hence, it is unclear from our data whether the infection-mediated systemic CMI is responsible for the declining numbers of Candida organisms and whether this acquired immunity would have more impact in a less dominant environment. It does appear, however, that some type of systemic and/or locally derived immune activity is expressed in the vagina during the infection, since mononuclear-like and polymorphonuclear-like cells associated with yeast mycelium have been observed in wet-mount preparations of lavage fluid from infected animals but not from PBS-treated animals (data not shown).

In summary, although the murine model of experimental Candida vaginitis has been used for several years to study biological interactions of Candida spp. and the efficacy of antifungal agents, it has never been carefully studied as a means of identifying the immune events associated with Candida vaginitis. The fact that systemic CMI reactivity is generated as a result of vaginal infection in mice and can be detected and reproducibly measured suggests that this model can be used to study the role of CMI as a possible defense mechanism in experimental Candida vaginitis. Accordingly, vaginal candidiasis can be studied in the presence of systematically derived Candida-specific DTH T cells to examine the protective role of systemic CMI for mucosal surfaces. Moreover, T-cell populations present in the local vaginal tissue during an infection and lymphokines produced at the systemic and local levels can be examined in detail. In relation to clinical RVVC, since susceptibility of RVVC patients to repeated episodes of vulvovaginal candidiasis has been associated with impaired CMI (16, 19, 41, 43, 44), results generated from the murine model of experimental vaginal candidiasis may provide insight into the role of CMI in infections of mucosal surfaces of the vagina.

REFERENCES


