Candida-Specific Antibodies during Experimental Vaginal Candidiasis in Mice

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Protective host defense mechanisms against vaginal Candida albicans infections are poorly understood. Although cell-mediated immunity (CMI) is the predominant host defense mechanism against most mucosal Candida infections, the role of CMI against vaginal candidiasis is uncertain, both in humans and in an experimental mouse model. The role of humoral immunity is equally unclear. While clinical observations suggest a minimal role for antibodies against vaginal candidiasis, an experimental rat model has provided evidence for a protective role for Candida-specific immunoglobulin A (IgA) antibodies. Additionally, Candida vaccination-induced IgM and IgG3 antibodies are protective in a mouse model of vaginitis. In the present study, the role of infection-induced humoral immunity in protection against experimental vaginal candidiasis was evaluated through the quantification of Candida-specific IgA, IgG, and IgM antibodies in serum and vaginal lavage fluids of mice with primary and secondary (partially protected) infection. In naïve mice, total, but not Candida-specific, antibodies were detected in serum and lavage fluids, consistent with lack of yeast colonization in mice. In infected mice, Candida-specific IgA and IgG antibodies were induced in serum with anamnestic responses to secondary infection. In lavage fluid, while Candida-specific antibodies were detectable, concentrations were extremely low with no anamnestic responses in mice with secondary infection. The incorporation of alternative protocols—including infections in a different strain of mice, prolongation of primary infection prior to secondary challenge, use of different enzyme-linked immunosorbent assay capture antigens, and concentration of lavage fluid—did not enhance local Candida-specific antibody production or detection. Additionally, antibodies were not removed from lavage fluids by being bound to Candida during infection. Together, these data suggest that antibodies are not readily present in vaginal secretions of infected mice and thus have a limited natural protective role against infection.

Vulvovaginal candidiasis (VVC) is a significant problem in women during their reproductive years. Candida albicans, a dimorphic fungus, is the causative agent in 85 to 95% of VVC cases (42, 43). C. albicans is both a commensal organism of the genital and gastrointestinal tracts and an opportunistic pathogen of the same tissues (34). Over 75% of women will experience at least one episode of acute VVC during their lifetime, and another 5 to 10% will experience recurrent VVC (RVVC) (42, 43). While acute VVC is usually associated with predisposing factors such as antibiotic or oral contraceptive usage, pregnancy, or diabetes (30, 42, 43), RVVC is idiopathic, with no known predisposing factors (42, 43). Rather, susceptibility to RVVC is thought to be associated with a local immune dysfunction or deficiency that allows the overgrowth of the commensal organism and subsequent conversion to the pathogenic form (16, 20). Understanding the natural protective response(s) against VVC is important in determining the immunological factor(s) that contribute to RVVC.

Cell-mediated immunity (CMI) through a Th1-type response is thought to be the predominant host defense mechanism against mucosal C. albicans infections (6, 38, 39). However, in women with RVVC, recurrent episodes occur in the presence of normal levels of systemic Candida-specific Th1-type CMI, suggesting that any dysfunction or deficiency is at the local level (16). Likewise, in the estrogen-dependent murine model of vaginal candidiasis, systemic Candida-specific Th1-type CMI plays little to no protective role (17, 19). Despite this, susceptibility of mice to C. albicans vaginitis upon rechallenge can be partially overcome by some form of locally acquired mucosal immunity (14, 15). However, a lack of changes in local vaginal T cells and a lack of infiltrating systemic T cells into the vagina during an infection suggest little to no protective role for systemic or local CMI (14). In fact, the reduction in T cells expressing homing receptors important for infiltration into mucosal tissues in the draining lymph nodes (22), together with high vaginal levels of the immunoregulatory cytokine, transforming growth factor β (3, 41, 46–48), suggests that some form of immunoregulation may be acting at the vaginal mucosa, precluding a more profound CMI response.

Investigations of innate immunity against vaginal Candida infections have shown that while polymorphonuclear leukocytes are often present in vaginal lavage fluids of infected mice, their presence does not correlate with reduced vaginal fungal burden (14, 40). Conversely, vaginal epithelial cells from mice, humans, and nonhuman primates have been shown to inhibit the growth of C. albicans in vitro (1, 44, 45). Although this has yet to be demonstrated in vivo, epithelial cells may play a role against C. albicans vaginal infections as an innate resistance mechanism.

The role of humoral immunity against vaginitis is uncertain. In patients with RVVC, the presence of normal or even ele-
vated levels of Candida-specific (Candida-binding) immunoglobulin A (IgA) and IgG antibodies in serum or vaginal secretions suggests little to no protective role for antibodies against vaginitis (12, 31, 32). In mice, however, Candida mannann-specific IgM and IgG3 antibodies have been shown to be protective against experimental systemic and vaginal Candida infections (23–25, 36). Additionally, in a rat model of vaginal candidiasis, vaginal infection-induced aspartyl proteinase-specific IgA antibodies contribute to protection against infection (5, 9, 10).

Based on these contrasting findings and evidence for some form of acquired protective response against secondary vaginal challenge in mice, we sought to determine whether anti-Candida antibodies are induced in vaginally infected mice and contribute to protection.

**MATERIALS AND METHODS**

**Mice.** Female CBA/J (H-2^d_) and BALB/c (H-2^b_) mice, 8 to 10 weeks of age (purchased from the National Cancer Institute, Frederick, Md.), were used throughout these studies. All animals were housed and handled according to institutionally recommended guidelines.

**Mouse infection.** (i) Primary infection. Mice were treated with estrogen by the subcutaneous injection of 0.02 mg of estradiol valerate (Sigma, St. Louis, Mo.) in 100 μl of sesame oil 72 h prior to inoculation with Candida and weekly thereafter. Vaginal inoculation was performed as previously described (17, 21) using 5 × 10^5 C. albicans 3153A blastoconidia in 20 μl of sterile phosphate-buffered saline (PBS) from a stationary-phase culture. Separate groups of mice were sacrificed on days 4, 10, and 17 postinfection. Serum was collected by retro-orbital bleeding prior to sacrifice, and vaginal lavage was performed as previously described (17, 21). Infection was confirmed by the enumeration of C. albicans by quantitative culture as previously described (17, 21). Following removal of the aliquot of lavage fluid for determination of fungal burden, the fluid was pooled by group and centrifuged at 800 × g, and the soluble fraction was collected and stored at −70°C.

(ii) Secondary infection. CBA/J mice were inoculated intravaginally in the absence of estrogen with 5 × 10^5 blastoconidia in 20 μl of sterile PBS, allowed 4 weeks to clear the low-grade infection, and given a second inoculation (5 × 10^5 blastoconidia) in the presence of estrogen as previously described. At the same time, another group of mice was inoculated for the first time (primary infection control) (15). Separate groups of 10 mice each were sacrificed at days 4 and 10 post-secondary inoculation. Serum and vaginal lavage fluid were collected, and vaginal fungal burden quantification was carried out as described above, as were vaginal lavage fluid processing and storage. In an alternative approach, CBA/J mice were inoculated with 5 × 10^5 blastoconidia in the presence of one dose of estrogen allowed 5 weeks to clear the high-grade infection, and then given estrogen again before being given a second inoculation (5 × 10^5 blastoconidia). Control mice with primary infection were similarly included. Groups of 10 mice each were sacrificed on the day of the secondary inoculation (day 0) and at days 4 and 10 postinoculation. Serum and vaginal lavage fluid were collected, and vaginal fungal burden was quantified as described above. Lavage fluid was also processed and stored as described above. Vaginal fungal burden was confirmed to be negative on the day of secondary inoculation.

**Total antibody quantification.** Total antibody concentrations were determined for serum and lavage fluids from mice with primary and secondary infection by enzyme-linked immunosorbtent assay (ELISA). Briefly, microtiter plates were coated with anti-mouse IgA, IgG, or IgM (10 μg/ml; Sigma) at 50 μl/well. After overnight incubation at 4°C, the plates were washed, and the nonspecific protein-binding sites were blocked with 10% fetal bovine serum (FBS) in PBS (blocking buffer) for 1 h at room temperature. Following washing, the samples (pooled serum or lavage fluid) and standards (purified mouse IgA, IgG, or IgM, serially diluted in PBS) (Sigma) were added in duplicate at 50 μl/well and incubated at room temperature for 2 h. Following washing, detection antibody (anti-mouse IgA, IgG, or IgM, conjugated to horseradish peroxidase [Sigma]), diluted 1:10,000 in blocking buffer, was added for 1 h at room temperature. Following the final washing, o-phenylenediamine dihydrochloride substrate (Sigma) was added. After incubation in the dark for 15 to 30 min, the absorbance was read at 450 nm on an automated plate reader (Labsystems, Helsinki, Finland), and antibody concentrations in the samples were extrapolated from the standard curve. Data were expressed as nanograms per milliliter.

**Candida-specific IgA, IgG, and IgM.** The procedure for Candida-specific ELISA was similar to that for total antibodies, except that the plates were coated with a Candida antigen, soluble cytoplasmic substances (SCS) (26, 33) (kindly provided by Judith Domer, Appalachian State University, Boone, N.C.), or heat-killed Candida blastospores (HKB). Briefly, microtiter plates were coated with either 50 μg of SCS/ml or 100 HKB/ml at 50 μl/well. The procedure then followed the same procedure as that for total antibody. Data were expressed as optical density (OD) at 450 nm.

**Concentration of lavage fluid.** Mice (CBA/J) were given a primary infection as described and sacrificed at day 17 postinfection. Lavage fluids were obtained from a total of 90 mice from three experiments and pooled. The pooled lavage fluids were then concentrated 10-fold by volume using a 10,000-molecular-weight exclusion membrane (Amicon, Danvers, Mass.). Protein in the sample was increased from 0.97 to 3.84 mg/ml (4–fold). Protein was quantified by the BCA protein assay (Pierce, Rockford, Ill.) with bovine serum albumin as the standard. The procedure was performed according to the manufacturer’s instructions, and plates were read at 595 nm on an automated plate reader (Labsystems). Data were expressed in milligrams per milliliter.

**Candida binding assay.** C. albicans blastospores were grown to stationary phase, and various concentrations of Candida—6 × 10^4, 3 × 10^4, 1.5 × 10^4, 5 × 10^3, 5 × 10^2, and 0 cells/ml—were incubated with purified rabbit anti-Candida IgG polyclonal antibody (Sigma), 1:2,000 dilution in sterile PBS for 1 h. Following incubation, samples were centrifuged, and the supernatants were removed. Supernatants were then tested in a Candida-specific IgG ELISA, using the same methods as above except that the detection antibody was anti-rabbit IgG conjugated to alkaline phosphatase and the substrate was p-nitrophenyl phosphate. The plates were read at 405 nm on an automated plate reader (Labsystems), and data were expressed as OD at 405 nm.

**Indirect fluorescent antibody staining.** Mice (CBA/J) were given a primary infection as described above and sacrificed at day 9 postinfection. Lavage fluids were obtained from a total of 20 mice. Lavage fluid (containing hyphae from infected mice) was incubated with blocking buffer (10% FBS in PBS) for 1 h at room temperature, which was followed by centrifugation and washing with sterile PBS. The samples then were incubated with biotin-conjugated anti-mouse polysaccharide immunoglobulin (Sigma) at a 1:1,000 dilution in sterile PBS for 1 h. Following incubation, samples were centrifuged, and the supernatants were removed. Supernatants were then tested in a Candida-specific IgG ELISA, using the same methods as above except that the detection antibody was anti-rabbit IgG conjugated to alkaline phosphatase and the substrate was p-nitrophenyl phosphate. The plates were read at 405 nm on an automated plate reader (Labsystems), and data were expressed as OD at 405 nm.

**RESULTS**

**Antibodies in naive mice.** In the first series of studies we sought to confirm that Candida-specific antibodies were not present in naive mice. For this, serum and vaginal lavage fluid from naive mice were evaluated for total antibodies and Candida-specific antibodies (to Candida SCS). Results showed that total IgA, IgG, and IgM antibodies were present in serum and vaginal lavage fluid of naive mice, while Candida-specific antibodies were undetectable (Fig. 1), consistent with the lack of yeast colonization in naive mice.

**Antibodies in serum of infected mice.** In the next series of studies, we sought to examine the presence of Candida SCS-specific IgA, IgG, and IgM in serum of mice with primary and secondary infection. In these experiments, it was confirmed that vaginal fungal burden in mice with secondary infection was significantly reduced compared to mice with primary infection at days 4 (P = 0.0055) and 10 (P = 0.0040) postinoculation (data not shown), as previously reported (15). Results of...
Candida-specific antibodies showed that IgA was increased in mice with secondary compared to primary infection at day 4 postinfection \((P < 0.006)\) (Fig. 2A), while IgG was increased at days 4 and 10 post-secondary infection \((P < 0.03)\) (Fig. 2B). In contrast, Candida-specific IgM was not detected in serum (data not shown). Results did not differ when mice were treated with estrogen once during the primary infection as a means to prolong the primary infection and increase antibody production (data not shown).

Antibodies in vaginal lavage fluid of infected mice. To examine antibodies present locally during infection, Candida SCS-specific IgA, IgG, and IgM were measured in vaginal lavage fluid of mice with primary and secondary infection. Results showed that Candida-specific antibody isotypes were low but detectable in lavage fluid of infected mice (Fig. 3), with no significant differences detected in Candida-specific IgA (Fig. 3A), IgG (Fig. 3B), or IgM (Fig. 3C) antibodies between mice with primary and secondary infection. Similar to serum antibody results, results did not differ in mice with secondary infection if the mice were treated with estrogen once during the primary infection (data not shown).

Antibodies in concentrated lavage fluid. Due to the low levels of Candida-specific antibodies in lavage fluid of infected mice, the fluid was concentrated 10-fold by volume and tested again for the presence of Candida SCS-specific antibodies. Protein content was increased approximately fourfold from 0.97 to 3.84 mg/ml. However, no significant differences in the amounts of Candida-specific antibody were detected in concentrated versus nonconcentrated lavage fluids (data not shown).

Candida-specific antibodies in BALB/cJ mice. To determine if the virtual lack of Candida-specific antibodies in vaginal secretions of infected mice was specific to the CBA/J \((H-2^k)\) strain of mice, Candida SCS-specific antibodies were measured in serum and lavage fluid of primary infected BALB/cJ mice \((H-2^d)\) (Fig. 4). In serum of primary infected BALB/cJ mice, increases in Candida-specific IgG \((P < 0.004)\) (Fig. 4A) and IgM \((P < 0.008)\) (Fig. 4B), but not IgA, were detected by days...
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detect total IgG, IgM, and IgA in both serum and lavage fluids of naïve mice. As expected, Candida-specific antibodies were not detected in naïve animals, consistent with the lack of yeast colonization in mice. We next determined whether Candida-specific antibodies were present in serum or vaginal lavage fluids of mice with primary infection and whether anamnestic responses could be detected in mice with secondary infection. Vaginal fungal burden in mice with primary and secondary infection followed the same pattern as previously reported (15); a persistent infection was observed in animals with primary infection, and a partial protection against infection was observed in animals with secondary infection. Although a systemic anamnestic delayed-type hypersensitivity response has been shown in partially protected mice with secondary infection (15), no evidence of a role for systemic or local CMI has been identified (15). Thus, it was possible that humoral immunity contributed to protection. The induction of Candida-specific IgA and IgG antibodies was detected in serum of mice with primary infection. Additionally, significant increases of serum Candida-specific IgG antibodies were detected in mice with secondary compared to primary infection, indicating a systemic humoral anamnestic response. This anamnestic humoral response (IgG) to a vaginal infection, however, was not similarly observed in the vagina, as no increases in Candida-specific IgG or IgA antibodies were detected in vaginal lavage fluid specimens from mice with secondary versus primary infection. In fact, Candida-specific IgA, IgG, or IgM antibodies were detected in low amounts in vaginal fluids during either infection. Thus, although Candida-specific IgG in serum could have been responsible for the partial protection in mice with secondary infection, the low levels of antibodies in vaginal secretions and lack of a local anamnestic effect makes this possibility unlikely. This is also consistent with the results of a previous study showing little evidence of migration of antibodies from the peripheral circulation into the vagina (13), although other studies would argue against this (24, 25). Vaginal-specific antibodies were equally unlikely to contribute to protection based on the low amounts detected. Although B cells are not required on-site for the presence of antibodies in mucosal washes, our results are consistent with the absence of B cells in the mouse vagina (35) and the lack of lymphocytes in vaginal lavage fluid specimens of infected mice (40). On the other hand, passive immunization by mannan-specific antibodies were equally unlikely to contribute to protection based on the low amounts detected. Although B cells are not required on-site for the presence of antibodies in mucosal washes, our results are consistent with the absence of B cells in the mouse vagina (35) and the lack of lymphocytes in vaginal lavage fluid specimens of infected mice (40). On the other hand, passive immunization by mannan-specific antibodies showed protection against vaginitis without detection of antibodies in vaginal fluids, presumably by antibody bound to antigen infiltrating the vaginal mucosa (25). Thus, it remains possible that the partial protection was due to undetectable systemic-derived antibodies bound to Candida antigen.

Assuming the lack of such undetectable systemically derived antibodies, there are several possibilities to explain the virtual absence of Candida-specific antibodies in the lavage fluids of infected or partially protected animals. One possibility was that the assays were not sensitive enough to adequately detect the antibodies. This concern could be eliminated from consideration, however, by the fact that concentrated lavage fluids showed no enhanced detection of Candida-specific antibodies. Another possibility was that the SCS antigen used for detection of the Candida-specific antibodies had an insufficient number of antigenic determinants to adequately detect a wide range of specificities (i.e., secretory aspartyl proteinases-specific anti-

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**FIG. 3.** Candida-specific IgA, IgG, and IgM in lavage fluid specimens from mice with primary and secondary infection. Lavage fluid specimens were collected from mice with primary (1°) and secondary (2°) infection at days 4, 10, and 17 (primary only) postinoculation, and pooled samples were assayed for Candida-specific IgA, IgG, and IgM to Candida SCS by ELISA. Mean amounts ± the standard errors of the means (error bars) of each antibody are shown expressed as specific OD at 450 nm with the OD of samples from naïve mice subtracted. Actual OD values ranged from 0.042 to 0.085. Data shown are cumulative of three experiments using 10 mice per group per time point for each experiment.
FIG. 4. *Candida*-specific antibodies in serum and lavage fluid specimens from BALB/cJ mice. Serum and lavage fluid were collected from mice with primary infection on day 4, 10, and 17 postinoculation, and pooled serum and vaginal lavage fluid samples were assayed for total antibodies and *Candida*-specific antibodies to *Candida* SCS by ELISA. Mean amounts ± the standard errors of the means (error bars) of each antibody are shown expressed as specific OD at 450 nm with the OD of samples from naïve mice subtracted. Actual OD values ranged from 3.2 to 3.5 (serum) and from 0.74 to 1.34 (lavage fluid). Data shown are cumulative of two experiments using 10 mice per group per time point.
bodies), despite SCS being the most reactive of several soluble Candida antigens in an ELISA (data not shown) and also one of the strongest stimulators of lymph node and peripheral blood lymphocyte proliferation (16, 18). This possibility was considered unlikely, however, on the basis of the similar detection of Candida-specific IgA and IgG in serum and lavage fluids from mice with primary and secondary infection using heat-killed Candida blastospores (HKB) as the capture antigen. Similar results were found for detection of Candida-specific antibodies in human saliva (49). Thus, it would appear that SCS contains a broad range of both cytoplasmic and cell surface antigenic determinants to survey a wide array of Candida-specific antibodies in biological fluids.

A third possibility was that the strain of mice used (CBA/J) (H-2k) was inadequate for Candida-specific antibody production. However, an identical analysis conducted with BALB/cJ (H-2b) mice, a strain of mice associated with Th2-type responses and antibody production (8, 11, 27, 28), showed similar results. A fourth possibility is that, due to infection with Candida, anti-Candida antibodies could be bound by Candida in the lavage fluid and removed during processing for the soluble fraction. In studies to address this, results of a Candida binding assay showed that while detection of Candida-specific antibody was reduced with high concentrations of Candida, only a 19% reduction was observed at the concentration of Candida that represented a Candida/antibody ratio found in infected mice. Additionally, no antibody was detected on Candida recovered from lavage fluids of infected mice, as a confirmation that Candida-specific antibodies were not being removed to a significant degree by the presence of Candida in the vagina. Finally, it was possible that suboptimal conditions were present for the induction of the primary antibody response in the absence of estrogen, thereby reducing the potential for local anamnestic responses following secondary challenge. To address this, mice were given the primary infection in the presence of one dose of estrogen in an attempt to extend the vaginal presence of Can-

FIG. 5. Candida-specific antibodies in serum and lavage fluids using HKB as the coating antigen. Serum and lavage fluid were collected from CBA/J mice with primary and secondary infection on days 4 and 10 postinoculation, and pooled serum and vaginal lavage fluid samples were assayed for Candida-specific IgA and IgG by ELISA using Candida HKB as the coating antigen. Mean amounts ± the standard errors of the means (error bars) of each antibody are shown expressed as specific OD at 450 nm the OD of samples from naïve mice subtracted. Actual OD values ranged from 0.073 to 0.176. Data shown are cumulative of two experiments using 10 mice per group per time point.
dida for antibody production. However, results showed similar levels of Candida-specific antibodies in the vaginal fluid 28 to 35 days after the primary infection as well as a lack of any local anamnestic responses during a secondary infection.

Taken together, there appears to be little to no local Candida-specific antibody production in mice during either primary or secondary vaginal Candida infection. Thus, there is little evidence that humoral immunity or CMI contributes to the partial protection in mice with secondary infection. While the protective effect is considered to be acquired, it is possible that the protection is innate and simply amplified by the secondary challenge. Anti-Candida activity by vaginal epithelial cells (1, 44) represents one such candidate.

One final consideration is the vast difference between the two rodent models regarding local Candida-specific antibody-mediated protection. One possibility is that aspartyl-proteinase-specific antibodies are not favorably induced in mice and that this contributes both to the low antibody production and lack of more significant protection in the mouse model. Another possibility is that some form of immunoregulation may be acting at the vaginal mucosa in mice that is postulated to preclude a more significant CMI response. This immunoregulation does not appear to be present in rats based on the strong T- and B-cell responses observed during experimental vaginitis (5, 9, 10). Thus, the putative CMI immunoregulatory mechanisms in mice may extend to humoral immunity as well. Such immunoregulation may also extend to innate resistance as demonstrated by the observation that neutrophils, cells with considerable anti-Candida activity in vitro (34), are ineffective against Candida in the vagina (2, 14). On the other hand, protective antimannan IgM and IgG3 antibodies given to mice results in protection against vaginitis, suggesting that if protective Candida-specific antibodies can be produced, they can be functional in the mouse vaginal microenvironment. In any event, the rat model appears to exemplify the immune events that should take place during an infection, based on protective responses to Candida at other mucosal sites (6, 7), while the mouse model appears to parallel the human condition, in which little to no evidence of adaptive protective responses is seen during episodes of acute VVC or RVVC. Alternatively, this may represent species-specific differences in the infection and may not be helpful for relating to the clinical condition.

Despite the results herein, the very fact that protective antibodies to Candida exist and can be effective in either animal

FIG. 6. Evaluation of Candida-specific antibodies on Candida from infected mice. Candida recovered from lavage fluid of day-9-infected animals was incubated with biotin-conjugated anti-mouse immunoglobulin followed by incubation with avidin-fluorescein isothiocyanate. Shown are bright-field (A and C) and fluorescent (B and D) images of hyphae from a mouse lavage fluid sample representative of 20 tested from separate mice (A and B) and of the positive control (Candida hyphae incubated with anti-Candida antibody) (C and D).
model provides a great deal of enthusiasm for the use of antibodies immunotherapeutically. According to Casadevall, "protective," "nonprotective," and "indifferent" antibodies comprise any pool of antigen-specific antibodies, with those present in the highest concentration dictating the role of humoral immunity in protection against infection (4). Thus, although antibodies are readily present in human vaginal secretions, concentrations of protective antibodies may not be present in high enough concentrations to be effective, whereas such protective antibodies appear to be abundant in rat vaginal secretions. Inasmuch as vast distinctions of antibody production and/or protection are evident in the experimental models or clinical setting, strides to formulate antibody-producing vaccines using an immunogen that induces protective antibodies would be extremely advantageous for the prevention of candidiasis in both immunocompetent and T-cell-immunocompromised individuals.

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