Clotrimazole Dampens Vaginal Inflammation and Neutrophil Infiltration in Response to *Candida albicans* Infection

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The pathology of vulvovaginal candidiasis (VVC) caused by *Candida albicans* is associated with a nonprotective inflammatory response and is frequently treated with clotrimazole. We investigated the mechanisms by which clotrimazole resolves VVC. Low levels of clotrimazole, which do not block fungal growth, inhibit expression of a “danger response” transcription factor, c-Fos, block production of proinflammatory cytokines, and inhibit neutrophil infiltration to the site of infection.

Candida albicans is a commensal resident of the human oral cavity, gastrointestinal tract, and vagina but can also cause both superficial infections and life-threatening disseminated candidiasis. Vulvovaginal candidiasis (VVC) is probably the most common form of *Candida* infection, as around 75% of women of childbearing age suffer from at least one episode of VVC, and a subset of women (5 to 10%) suffer from recurrent VVC (1).

In contrast to other forms of candidiasis, which are often associated with immune deficiencies or other predisposing factors, VVC frequently occurs in otherwise healthy individuals. Indeed, rather than being protective, an inflammatory response and associated neutrophil infiltration are thought to contribute to symptomatic VVC (2). This form of nonprotective inflammation is mediated by the S100A8 and S100A9 alarmins (also known as calprotectin), in addition to classical inflammatory mediators such as cytokines (3).

Clotrimazole is an azole antifungal commonly used to treat VVC. We have recently shown *in vitro* that clotrimazole can effectively inhibit *C. albicans*-induced lysis of vaginal epithelial cells at drug concentrations that do not block fungal growth or invasion into epithelial cells (4).

However, as symptomatic VVC is dependent largely on inflammation and neutrophil infiltration, the host response to fungal cells plays a critical role in the clinical outcome of VVC. The transition of *C. albicans* from commensal to pathogen is thought to be associated with hypha formation and increased fungal burden (5). Epithelial cells recognize this event via prolonged mitogen-activated protein kinase (MAPK) signaling, resulting in activation of the transcription factor c-Fos via p38 and the MAPK regulatory phosphatase MKP1 via extracellular signal-regulated kinase 1/2 (ERK1/2). This “danger response” mechanism drives the expression of cytokines, which ultimately serve to recruit immune cells, such as neutrophils, to the site of infection (6).

In the current study, we sought to elucidate whether low-dose azole treatment may contribute to the resolution of VVC by preventing *C. albicans* from eliciting a proinflammatory response. We demonstrate that neutrophils are rapidly recruited to the supernatants of *C. albicans*-infected vaginal epithelial cells and that clotrimazole treatment blocks this infiltration. Moreover, our data suggest that inhibition of neutrophil migration may be mediated by dampening c-Fos expression and downregulation of proinflammatory cytokines.

Infections of vaginal epithelial cell line A431 with *C. albicans* strain SC5314 in the absence or presence of 1 μ M or 10 μ M clotrimazole (Bayer AG) were performed as previously described (4). For all experiments, 2.5×10^5 A431 cells were seeded into 6-well tissue culture plates and grown to confluence over 2 days in RPMI (PAA) supplemented with heat-inactivated fetal bovine serum (PAA).

To measure c-Fos expression, monolayers were infected with 3×10^6 yeast cells for 3 h, and RNA was extracted using an RNeasy kit (Qiagen). cDNA synthesis and real-time PCR were performed as described previously (7). Primers for the c-Fos (FBJ murine osteosarcoma viral oncogene homolog), YWHAZ (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide), and GAPDH genes were selected from PrimerBank (<http://pga.mgh.harvard.edu/primerbank/>) or RT-Primer-DB (<http://www.rtpriimerdb.org/index.php>) (see Table S1 in the supplemental material). Relative c-Fos transcript abundance was determined using the cycle threshold ($\Delta\Delta C_T$) method (8) using the transcript levels of the YWHAZ and GAPD housekeeping genes as internal controls. The experiment was performed in duplicate three times.

For cytokine determination, A431 monolayers were infected with 2×10^3 yeast cells for 24 h. Levels of interleukin 1 α (IL-1 α), IL-6, and IL-8 in the supernatant were measured by enzyme-linked immunosorbent assay (ELISA) (eBioscience) and normalized to an uninfected control. Experiments were performed in duplicate at least three times.

For determining neutrophil migration, A431 monolayers were infected with 2×10^3 yeast cells in RPMI medium without phenol red (PAA) for 24 h. Supernatants were filter sterilized, and 600 μ l was added to the lower compartment of a Transwell plate (Corning; 6.5-mm-diameter inserts, 3- μ m pore size). Neutrophils were

Received 13 June 2013 Returned for modification 13 July 2013

Accepted 21 July 2013

Published ahead of print 29 July 2013

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AAC.01244-13>.

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[doi:10.1128/AAC.01244-13](http://dx.doi.org/10.1128/AAC.01244-13)

FIG 1 Clotrimazole inhibits *C. albicans*-induced stimulation of the danger response in vaginal epithelium. A431 vaginal epithelial cells were infected with wild-type *C. albicans* (SC5314) for 3 h (A) or 24 h (B to D) in the absence or presence of 1 or 10 μ M clotrimazole. c-Fos gene expression was determined via quantitative reverse transcription (RT)-PCR and normalized against an uninfected control (A). The experiment was performed three times. IL-1 α (B), IL-6 (C), and IL-8 (D) levels were assessed by ELISA and normalized against an uninfected control. The experiment was performed at least three times. Asterisks indicate statistical significance ($P < 0.05$) by ANOVA.

isolated and fluorescently labeled as previously described (9, 10), and 5×10^5 neutrophils in 100 μ l was added to the upper compartment of the Transwell. Following 1 h of incubation, 100 μ l was removed from the lower compartment, and fluorescence was determined at 485/530 nm. Values for clotrimazole-treated and untreated *C. albicans*-infected monolayers were normalized against uninfected monolayers. The experiment was performed in duplicate three times.

Blood was obtained from healthy human donors with written informed consent. The blood donation protocol and use of blood for this study were approved by the institutional ethics committee (Ethik-Kommission des Universitätsklinikum Jena, permission no. 2207-01/08).

Statistical analyses were performed using GraphPad: Student's *t* test (for single comparisons) or one-way analysis of variance (ANOVA) (for multiple comparisons). *P* values of <0.05 were considered significant.

To analyze the impact of low levels of clotrimazole on the host response to *C. albicans* infection, we used 1 or 10 μ M. Such low concentrations of clotrimazole do not block hypha formation, and clotrimazole-treated hyphae are able to adhere to and invade into vaginal epithelial cells at rates comparable to those of untreated fungal cells (4, 11). Despite initial invasion, clotrimazole-treated *C. albicans* is unable to subsequently disseminate throughout the epithelium and does not lyse host cells (4).

Vaginal epithelial cells recognize both noninvasive yeast and invasive hyphae via ERK1/2 signaling, whereas activation of c-Fos is specifically associated with hypha formation and the damage response (12). We first analyzed phosphorylation of ERK1/2 in response to *C. albicans* infection in the absence and presence of clotrimazole after 15 min and 2 h. Clotrimazole did not affect ERK1/2 phosphorylation (data not shown), indicating that, upon treatment, *C. albicans* was still recognized by the vaginal epithelium and that the ERK1/2 MAPK pathway remained responsive.

We next analyzed expression of the c-Fos transcription factor. Infection of vaginal epithelial cells with *C. albicans* resulted in robust upregulation of c-Fos expression (Fig. 1A). However, treatment of *C. albicans* with clotrimazole at 1 or 10 μ M blocked *C. albicans*-induced upregulation of c-Fos, with levels remaining comparable to those of uninfected control cells (Fig. 1A, dotted line).

As c-Fos drives the expression of proinflammatory cytokines, we next tested production of IL-1 α , IL-6, and IL-8 by vaginal epithelium following *C. albicans* infection. Figures 1B to D show that treatment of *C. albicans* with clotrimazole reduced production of all three proinflammatory cytokines, although for IL-6 the difference was not statistically significant.

As clotrimazole inhibited *C. albicans*-induced expression of c-Fos and the production of proinflammatory cytokines, particularly IL-8, we postulated that azole treatment may also reduce neutrophil recruitment to the site of infection (13). The supernatants of vaginal epithelial cells, infected with *C. albicans* in the absence or presence of clotrimazole, were added to the lower compartment of a Transwell plate. Fluorescently labeled neutrophils were added to the upper Transwell compartment, and migration to the lower compartment was determined by measuring fluorescence.

Figure 2 shows that the supernatant of vaginal epithelium infected with *C. albicans* was a strong inducer of neutrophil recruitment, whereas the supernatant of vaginal epithelium infected with *C. albicans* in the presence of clotrimazole attracted significantly reduced numbers of neutrophils (indistinguishable from that of the uninfected control; Fig. 2, dotted line). This suggests that, in this model system, clotrimazole treatment can block the infiltration of neutrophils to the site of *C. albicans* infection.

Neutrophils play pivotal roles in controlling *Candida* infections (6, 14, 15). However, in the case of experimental vulvovaginal candidiasis in mice, neutrophils are not protective but rather contribute to symptomatic disease (16). Although low-level clotrimazole treatment blocks epithelial lysis by *C. albicans* *in vitro* (4), it is unclear what effect this has on the host response. Here, we report that clotrimazole blocked c-Fos upregulation and proinflammatory cytokine production by vaginal epithelial cells in response to *C. albicans* infection. Importantly, *C. albicans*-induced neutrophil infiltration was abrogated by treatment with clotrimazole.

Untreated *C. albicans* rapidly form hyphae following contact with epithelial cells. These hyphae adhere to and then invade the epithelium (4), and these events in turn result in activation of an epithelial "danger response" pathway, mediated by c-Fos (5, 12).

Interestingly, despite blocking *C. albicans*-induced epithelial cell lysis, clotrimazole treatment does not block hypha formation,

FIG 2 Clotrimazole blocks neutrophil infiltration to *C. albicans*-infected vaginal epithelium. A431 vaginal epithelial cells were infected with wild-type *C. albicans* (SC5314) for 24 h in the absence or presence of 10 μ M clotrimazole. The supernatants were then added to the lower compartments of a Transwell plate. Fluorescently labeled neutrophils were added to the upper compartments, and neutrophil migration was determined by measuring fluorescence of the medium in the lower compartment. Results for control infection and infection in the presence of clotrimazole were normalized against values from uninfected control monolayers (dotted line). The experiment was performed three times. Asterisk denotes *P* values of <0.05 by Student's *t* test.

nor does it prevent initial invasion into vaginal epithelial cells. Indeed, clotrimazole-treated *C. albicans* invades vaginal epithelial cells at the same rate as untreated fungal cells during the first 3 h of infection (4). Therefore, we demonstrate an additional novel finding that, under the conditions tested, *C. albicans* hyphae can invade vaginal epithelial cells without triggering c-Fos activation or the production of proinflammatory cytokines. In this context, it appears that the (vaginal) mucosal immune system has evolved to tolerate moderate levels of epithelial invasion by *C. albicans*, in addition to tolerating noninvasive yeast cells (5).

Our data have direct clinical relevance, suggesting that clotrimazole treatment may prevent VVC-associated damage via two mechanisms: first, by blocking direct fungus-mediated damage, by inhibiting prolific hyphal dissemination within the epithelium (4); second, by preventing fungal activation of the epithelial “danger response,” clotrimazole treatment may dampen local inflammation and reduce neutrophil infiltration. It should be noted that clotrimazole has also been shown to inhibit tumor necrosis factor alpha (TNF- α)-induced IL-8 production and NF- κ B activity in the absence of fungal cells (17), suggesting that this azole may have additional anti-inflammatory actions.

In conclusion, we propose that resolution of VVC by clotrimazole treatment may be associated with inhibition of both pathogen- and host-induced damage.

(Results from this study were presented at the Gordon Research Conference—Immunology of Fungal Infections, January 2013, Galveston, TX.)

ACKNOWLEDGMENTS

This work was supported by Bayer Laboratories and the resources and facilities at the Hans Knoell Institute (HKI) Jena, Germany. D. Wilson and B. Hube were supported by the ERA-NET PathoGenoMics Program (Candicol; BMBF 0315 901 B). P. Miramón and B. Hube were supported by the European Commission through the FINSysB Marie Curie Initial Training Net-

work (PITN-GA-2008-214004). B. Hebecker, N. Jablonowski, and B. Hube were supported by the Center for Sepsis Control and Care (CSCC), BMBF FKZ 01EO1002. J. R. Naglik and D. L. Moyes were funded by the Medical Research Council (MR-J008303-1) and Biotechnology and Biological Sciences Research Council (BB-J016411-1). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

There are no transparency declarations to make.

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