

Local immune responsiveness following intravaginal challenge with *Candida* antigen in adult women at different stages of the menstrual cycle

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Recurrent vulvovaginal candidiasis (RVVC) is a significant problem in women of childbearing age and is most often caused by *Candida albicans* that asymptotically colonizes mucosal tissues. Although some form of local immune dysfunction is postulated to precipitate bouts of RVVC, the normal protective vaginal host response to *C. albicans* is poorly understood. In an effort to stimulate the natural adaptive response to yeast in healthy women without a history of VVC, commercial *Candida* skin test antigen was introduced intravaginally and changes in cytokines/immunomodulators were monitored in vaginal lavage fluid pre- and post-antigen challenge. In an earlier pilot study using small numbers of women without controlling for stages of the menstrual cycle, we reported elevated cytokines in vaginal secretions of antigen challenged women. The present study, however, that employed a similar design in a large number of women during each stage of the menstrual cycle showed no evidence of local immune stimulation, including changes in Th and proinflammatory cytokines, IgE, histamine, and prostaglandin, despite a natural modulation of vaginal cytokines over the course of the menstrual cycle. Taken together, these results suggest that either some form of vaginal immunoregulation/tolerance is evident in response to yeast or that more advanced clinical designs are required to detect the normal protective vaginal response to *C. albicans*.

Keywords recurrent vulvovaginal candidiasis; vaginal immunity; *Candida albicans*; cytokines; menstrual cycle

Introduction

Vulvovaginal candidiasis (VVC) is a common mucosal infection that affects an estimated 75% of women at least once during their reproductive years [1]. *Candida albicans*, a commensal fungal organism of the gastrointestinal and female genitourinary tracts, is the causative agent in 85–90% of all VVC cases [2]. Although most women experience infrequent episodes of VVC precipitated by pregnancy, use of oral contraceptives,

uncontrolled diabetes mellitus, and antibiotic usage, 5–10% of otherwise healthy women suffer from recurrent VVC (RVVC) (more than three episodes per annum) without any recognizable predisposing factors [3]. Rather, an undefined immune deficiency or dysfunction in the normal host protective mechanisms is postulated to be responsible for RVVC [2,4,5]. Although T helper (Th) 1-type cell-mediated immunity (CMI) in the peripheral blood is considered the predominant host defense mechanism against mucosal *C. albicans* infections [68], several clinical studies have demonstrated that most women experience RVVC despite normal levels of *Candida*-specific Th1-type CMI in the peripheral circulation [9–12]. Studies in animal models also support a lack of any role for *Candida*-specific systemic CMI in protection against vaginal *Candida* infections [13–15].

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To date the role of local CMI has equally been challenged. In experimental infections in mice, phenotypically distinct vaginal-associated T cells do not change in percentage or composition during either primary or secondary vaginal infections and with no evidence for systemic T cell infiltration [16]. Furthermore, local cytokines in naïve and infected mice are predominated by transforming growth factor-beta (TGF- β), a potent down-regulatory cytokine, with little to no expression of other Th1/Th2 cytokines [17]. We recently reported that although the appropriate cell adhesion molecules are upregulated on the murine vaginal endothelium during a vaginal infection to support T cell infiltration, T cells with the reciprocal adhesion molecules become reduced rather than accumulate in the draining lymph nodes [18], providing a potential explanation for the lack of cellular infiltration into the vagina during an infection. Together, these results suggested that some form of immunoregulation may prohibit a more profound cell-mediated response during a vaginal *Candida* infection.

Clinically, a large study was conducted that examined Th1/Th2 cytokines in vaginal lavages of women with RVVC. These women who were either symptomatic at the time of testing or in remission and culture negative, showed no distinct pattern of differences in cytokines compared to control women with no history of vaginitis that could account for susceptibility to RVVC [19]. Studies examining *Candida*-specific antibodies in vaginal secretions also failed to show a deficiency that would contribute to susceptibility to RVVC [20]. In the former, it was reasoned that due to unavoidable circumstances in the design (i.e., dynamics of infection, specificity of the cytokine expression, influence of the menstrual cycle), specific deficiencies may not have been realized. To circumvent this, a set of pilot studies using small numbers of women was conducted that involved intravaginal challenge of *Candida* antigen to normal healthy women without a history of vaginitis to identify what type of immune reactivity is stimulated in response to antigen. It was reasoned that since these control women were protected against infection, an adaptive response detected by differences in cytokine and/or *Candida*-specific antibodies in vaginal lavages between pre and post-challenge would be demonstrable. Although intravaginal administration of heat-killed *C. albicans* with or without a gelatin suppository did not stimulate evidence of responses, intravaginal challenge with *Candida* skin test antigen, without reference to stage of the menstrual cycle, showed some evidence for elevated Th1-type cytokines 16–18 h post antigen administration [19] consistent with resistance to *Candida* infection [21].

The purpose of the present study was to conduct a large study using a similar intravaginal antigen challenge

during distinct stages of the menstrual cycle in order to confirm or deny these pilot results and to evaluate the influence of reproductive hormones on any local responsiveness.

Materials and methods

Subjects

Healthy women ($n = 105$) between the ages of 18 and 55 years of age, non-pregnant, not on any form of hormonal contraception, and with no known immunological disorders were enrolled. Eligible subjects were enrolled through the Obstetrics and Gynecology Clinic at the Medical Center of Louisiana at the Louisiana State University Health Sciences Center, New Orleans. Informed consent was obtained from all participants and all participants/patients and all procedures were followed in the conduct of clinical research in accordance with the Institutional Review Board at the Louisiana State University Health Sciences Center, New Orleans

Specimen collection and processing

Specimens collected from the subjects included blood (30 ml) by venipuncture, a vaginal swab, a vaginal smear, and a vaginal lavage performed with 5 ml of non-pyrogenic sterile saline. The lavage fluid was collected after a 30–40 sec aspiration. The vaginal swab was streaked onto *Candida* chromagar (CHROMagar, Paris, France) plates, and incubated at 37 °C for 48 h. Green colonies were confirmed as *C. albicans* if positive for germ tube formation in fetal bovine serum (FBS) incubated for 3–4 h in a 37 °C water bath. All other colonies were defined as non-*C. albicans* yeast. Thirteen percent of subjects had demonstrable asymptomatic vaginal yeast colonization with 67% of the isolates identified as *C. albicans*. The vaginal smear was spray fixed and stained with the Papanicolaou stain (pap smear). Cervical mucus present in any lavage fluid was solubilized with 100 mM dithiothreitol prior to processing in order to release any immunomodulators that can become sequestered [22]. The lavage fluid was processed by centrifugation followed by sterile filtration through a 0.45 μ m membrane, and frozen in aliquots of 1.5 ml at –70 °C until use. Total protein content in each lavage fluid was quantified by the BCA commercial protein assay kit (Pierce, Rockford, IL). Peripheral blood lymphocytes (PBL) were isolated from whole blood using Ficoll Paque (Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation as previously described [12]. Serum was collected and used for reproductive hormone analysis.

Clinical Protocol

Eligible women were asked to return to the clinic at a specific time during their menstrual cycle. Women tested during the follicular phase were instructed to return for testing within 3 days following the end of menstruation. For the ovulatory stage, the subject was given a luteinizing hormone (LH) surge kit (urine-based) (Ovu-quick) and asked to return within 24 h of showing a surge in LH as evidence of periovulation. Women tested during the luteal phase were instructed to return to the clinic between day 16–22 of the menstrual cycle (10–16 days following the end of menses). Participants were stratified into one of four groups of 10–12 women each. Two groups received sterile saline/0.5% human serum albumin (to account for the protein in the antigen prep) intravaginally, while the other two groups received *Candida* skin test antigen (Candin) (Alk) intravaginally. The saline or antigen was placed in a syringe kit. In each protocol, following the collection of baseline specimens (blood, vaginal swab, vaginal smear, and vaginal lavage), the participant was given instructions to administer the contents of the syringe into the back wall of the vaginal lumen at night before retiring. A plastic transfer pipette connected to the syringe enabled the contents to be inserted appropriately. The evening protocol was employed to avoid leakage. A feminine pad was used to collect any leakage with the participant providing the diameter of any evidence of leakage on the pad at their subsequent clinic visit. Diameters were scored as 0 to +4. For each protocol, the participant was blinded to the contents contained in the syringe kit. All participants were asked to abstain from sexual intercourse during the testing period. Of those women given saline or antigen, one group of women were instructed to return to the clinic 16–18 h post vaginal administration, and the other group 36–40 h post vaginal administration, for a second collection of blood and vaginal lavage, swab, and smear. Eleven women were tested with antigen or saline at each stage of the cycle over a 1–2 year period.

Vaginal cuff tissue

Non-diseased vaginal cuff material (pathology confirmed) was obtained from patients undergoing hysterectomy or vaginal repair surgery. The vaginal tissue was homogenized in a Triton-x homogenization buffer with protease inhibitors and the soluble fraction collected as previously described [17] and stored at -70°C until use in immune assays.

Systemic immune reactivity

Peripheral blood lymphocytes (PBL) from each subject were cultured *in vitro* for lymphoproliferation in

response to *Candida* antigens and the mitogen phytohemagglutinin (PHA) (Sigma, St. Louis, MO). Two *C. albicans* antigens were employed. *C. albicans* heat-killed blastoconidia (HKB) were prepared by incubating a fresh stationary phase blastoconidial culture of *C. albicans* 3153A for 2–3 h at 60°C . Cells were washed twice with PBS and stored at 4°C . *C. albicans* soluble cytoplasmic substances (SCS) was a kind gift of Judith Domer, Appalachian State University, Boone, NC. Proliferation assays were used as a general measure of *Candida*-specific systemic lymphocyte responsiveness. PHA was included as a quality control for the lymphocyte culture. The methods used in our laboratory for proliferation assays using PBL have been described in detail elsewhere [12,19]. Briefly, 2×10^6 PBL ml^{-1} were cultured in triplicate in sterile 96-well tissue culture plates (Costar, Corning, NY) in a total volume of 200 μl . Cells were incubated at 37°C , 5% CO_2 with serum-free AIM V lymphocyte medium (GIBCO) supplemented with 100 mM L-glutamine or with AIM V/L-glutamine medium containing 5×10^6 *C. albicans* HKB ml^{-1} , 125 $\mu\text{g ml}^{-1}$ *C. albicans* SCS, or 20 $\mu\text{g ml}^{-1}$ PHA. On the third (mitogen) or sixth (antigens) day of culture, 1.0 μCi of ^3H -thymidine (ICN Radiochemicals, Costa Mesa, CA) was added to each well and incubated for an additional 6 h. The contents of the wells were harvested onto glass fiber filters, and the radioactivity incorporated into the DNA was detected by liquid scintillation (Beckman Instruments, Mountainview, CA). Data were expressed as proliferation indices (mean counts per minute for stimulated cultures/mean counts per minute for unstimulated cultures).

Identification of stage of the menstrual cycle

Progesterone in sera was analyzed by radioimmunoassay (RIA) at the Clinical Endocrinology Laboratory in the Division of Reproductive Endocrinology at the Detroit Medical Center, Detroit, MI. Cellular maturation indices were evaluated on Papanicolaou-stained vaginal smears using the percentage of basal, intermediate, and superficial cells in several fields.

Immunological assays

The vaginal lavage fluids were assessed for Th1- and Th2-type and proinflammatory cytokines, total and *Candida*-specific IgE antibodies, histamine, and prostaglandin E_2 . The Th1-type cytokines included IL-2, IFN- γ , and IL-12. The Th2-type cytokines included IL-4, IL-10, and TGF- β . The proinflammatory cytokines included IL-1 α , IL-6, and TNF- α . Concentrations of immune mediators in lavage fluid were normalized to total protein. Comparisons were made between the concen-

trations of immunomodulators in initial versus final lavage. Results for all groups were expressed as median responses as a function of time between antigen/saline introduction and the second vaginal lavage.

Cytokines

Cytokines were quantified by ELISA using commercial capture and biotinylated detection antibodies (BD Pharmingen Corp. San Diego, CA) and the respective recombinant human cytokine as the standard. A standard curve for each assay was generated in sterile phosphate-buffered saline (PBS) to obtain the identical matrix of the lavage fluid. The assays were performed in EIA/A2 96-well plates according to manufacturer's instructions. Briefly, the plates were coated with the capture antibody ($2 \mu\text{g ml}^{-1}$) in coating buffer (0.1 M NaHCO_3) overnight at 4°C . The plates were washed 4 times with PBS/0.05% tween-20 (wash buffer). The non-specific protein sites were blocked with PBS/10% FBS (blocking buffer) for 2 h at room temperature. After the plate was washed several times the standard and samples were added to each well in duplicate using a volume of $50 \mu\text{l}$ and incubated for 2 h at room temperature. Following this incubation, the plate was washed again several times and the biotinylated detection antibody plus streptavidin-horseradish peroxidase (HRP) (both diluted 1:250) was added in a volume of $50 \mu\text{l}$ /well and incubated 1 h followed by the addition of the substrate (OPD) (Sigma). The absorbance values and subsequent quantification of each cytokine in pg/ml were analyzed using a Ceres 900 automated microplate reader (Bio-Tek Corp., Winooski, VT) and Kineticalc software (Bio-Tek). Data were expressed as pg cytokine/mg protein in the lavage fluid.

Total IgE

A sandwich ELISA assay was performed in 96 well EIA/A2 plates. Briefly, anti-human IgE ($10 \mu\text{g ml}^{-1}$) antibody (capture antibody) (Dako Corp., Carpinteria, CA) diluted in coating buffer (0.1 M NaHCO_3) was added to each well in a volume of $50 \mu\text{l}$ and incubated overnight at 4°C . On the following day, the plate was washed 4 times with wash buffer (PBS + 1% BSA, 0.05% Tween 20), and nonspecific binding sites were blocked with $100 \mu\text{l}$ /well blocking buffer (PBS + 3% BSA, 0.05% Tween 20) for 1 h at room temperature. After the plate was washed 4 times, samples were added in duplicate in a volume of $50 \mu\text{l}$ /well and incubated for 2 h at room temperature. The standard consisted of a commercially available purified anti-human IgE (Sigma) that was serially diluted from 1000 ng ml^{-1} . Preliminary studies revealed that undiluted samples were required for IgE determinations. At the conclusion of the incubation

period, the wells were washed 4 times, and alkaline phosphatase-conjugated anti-human IgE 1:1000 (all from Sigma), was added to each well in a volume of $50 \mu\text{l}$ and incubated for 1 h at room temperature. After washing 4 times, $50 \mu\text{l}$ of the pNPP substrate (Sigma) was added to each well and the reaction was allowed to develop for 2060 min. Quantification of IgE was conducted with the Ceres 900 microplate reader and Kineticalc software as described above. Results were expressed as $\text{ng } \mu\text{g}^{-1}$ protein.

Candida-specific IgE

Candida-specific IgE was quantified by ELISA using the same basic methodology as described above with the exception of the capture procedure and the standard. In this assay, instead of employing a capture antibody, *C. albicans* culture filtrate antigen (CaCF) [23] diluted to $10 \mu\text{g ml}^{-1}$ in coating buffer was used to coat the plates. Since there was no commercially available human Candida-specific IgE to employ as the standard, we used a pooled sample of all lavage fluids that showed positive absorbances (at least 2-fold above the mean absorbance of saline alone in a separate assay). The pooled sample was serially diluted (2-fold) from an arbitrary 1000 Units/ml to 15 Units/ml in PBS and added in a volume of $50 \mu\text{l}$ to separate wells. The blank wells contained $50 \mu\text{l}$ of PBS. Preliminary studies showed that undiluted samples were required for Candida-specific IgE determinations.

Histamine

Histamine in lavage fluid was quantified by a competitive enzyme immunoassay kit (Immunotech International, Westbrook, ME). To an anti-histamine antibody-coated microtiter plate, an acetylcholinesterase-histamine conjugate was added to each well together with either an acylated sample, or known concentrations of acylated histamine standard diluted from 5 ng ml^{-1} . Following an 18 h incubation, the plate was washed and the substrate was added to reveal the binding of any esterase-conjugated histamine to the antibody-coated plate. Quantification of histamine was determined using the Ceres 900 automated plate reader. Results in each sample were expressed as ng histamine/mg protein.

Prostaglandin E2 (PGE₂)

PGE₂ in the vaginal lavage fluid was quantified by a competitive enzyme immunoassay kit (Cayman Chemical, Buckinghamshire, England) whereby a constant concentration of acetylcholinesterase-conjugated PGE₂ competes with cold PGE₂ in the sample for binding to mouse anti-human PGE₂ antibodies (IgG). PGE₂ was first extracted and purified by affinity chromatography using an Amprep column (Amersham, Buckingham-

shire, England). Following the competitive reactions, the antibody-PGE₂ complex was captured onto a microtiter plate precoated with goat anti-mouse IgG antibodies in an 18 h assay. After washing the plate, the amount of peroxidase-labeled PGE₂ bound to the antibody-coated plate was determined colorimetrically by the Ceres 900 plate reader. Concentrations of PGE₂ in the samples were quantified from a standard curve generated from unlabelled PGE₂ serially diluted from 1000 pg ml⁻¹. Concentrations were expressed as pg PGE₂ mg⁻¹ protein.

Statistical analysis

Comparison of data among study groups was done with the non-parametric Mann-Whitney U test or parametric Student's *t* test. Significance was defined as *P* < 0.05 using a two-tailed test.

Results

Systemic immune responses

All enrolled subjects were evaluated for systemic responsiveness to *Candida* by means of a test for PBL proliferation to particulate and soluble *Candida* antigens, including heat-killed blastoconidia and soluble cytoplasmic substances. Results showed that 100% of women tested at the follicular and luteal phase and 94% of women tested at the ovulatory phase responded to one or both *Candida* antigens. Positive responses were defined as at least a 3-fold increase in tritiated thymidine uptake in antigen-containing PBL cultures compared to cells cultured in medium alone. Proliferation indices in positive cultures ranged from 3.4 to 15.7 with background tritiated thymidine uptake ranging from 500–1500 counts per minute (cpm). Patterns of responses did not differ depending on whether or not the women were asymptotically colonized with yeast as detected by swab culture (~13%) and were not appreciably different pre- or post- intravaginal administration. Responses were consistent in women tested longitudinally at each stage of the menstrual cycle (*n* = 11).

Local immunity in women challenged intravaginally with *Candida* antigen

Previously we reported results from a small pilot study, without reference to the stage of the menstrual cycle, showing that Th1-type cytokines were elevated 16–18 h following intravaginal introduction of *Candida* skin test antigen but not following administration of sterile saline [19]. The present study sought to confirm these findings in a large number of women with testing conducted at

distinct stages of the menstrual cycle to evaluate any influences of reproductive hormones. For this, at each of the three stages of the menstrual cycle, groups of 12 women each were evaluated for stimulation of vaginal immunity 16–18 or 36–40 h following intravaginal administration of *Candida* skin test antigen compared to sterile saline. Comparisons of immunomodulators in pre- and post-lavages normalized to total protein were used to identify effects of the local presence of antigen versus saline. The first series of studies were conducted during the follicular phase of the menstrual cycle employing the *Candida* antigen at a 1:10 dilution and evaluated immunomodulators in vaginal secretions 16–18 h post-challenge to reproduce the experimental design of the previous pilot study [19]. In contrast to the previous results, there was no evidence of changes in Th1, Th2, or proinflammatory cytokines, total or *Candida*-specific IgE, histamine, or PGE₂ in subjects given antigen compared to those given saline alone (data not shown). Additionally, no clinical evidence of vaginal inflammation was observed by common signs/symptoms (i.e., erythema, edema, itching, burning). We next conducted a similar design using undiluted antigen and with immunomodulators evaluated 16–18 h or 36–40 h post-challenge again during the follicular phase of the menstrual cycle. Results, however, remained unchanged with few significant differences in immunomodulators (Th1 [IL-2, IFN- γ , IL-12], Th2 [IL-4, IL-10, TGF- β], and proinflammatory [IL-1 α , IL-6, TNF- α] cytokines, total or *Candida*-specific IgE, histamine, and PGE₂) and no clinical signs/symptoms of inflammation or responsiveness in women given antigen compared to saline. This included no evidence of cellular infiltration by PMNs or lymphocytes in the vaginal lavages. These results did not differ between the ovulatory and luteal stage of the menstrual cycle. In fact, of the large panel of tests performed, the only significant differences based on median concentrations of each immunomodulator in pre- vs. post-lavage fluid were elevated IL-12 16–18 h post-antigen administration during the ovulatory stage (*P* < 0.032), and elevated IL-10 36–40 h post-antigen administration during the luteal phase (*P* < 0.035). There was very little evidence of any antigen leakage with only 8% (*n* = 7) of subjects showing any evidence of leakage with a rating of no more than +1 out of a possible +4 if present. Table 1 shows the serum estrogen, progesterone, and local cellular maturation indices as confirmation of stage of the menstrual cycle. Figures 1–3 show results for two Th1-type (IL-12, IFN- γ) (Figure 1), two Th2-type (IL-10, TGF- β) (Figure 2), and two proinflammatory (IL-1- α , IL-6) (Figure 3) cytokines, respectively, at each stage of the menstrual cycle. Figure 4 shows representative results of total IgE and histamine.

Table 1 Confirmation of stage of the menstrual of enrolled participants

Phase of menstrual cycle	N*	Estimated mean day of cycle**	Serum progesterone† ng ml ⁻¹ ± SEM	% superficial epithelial cells‡
Follicular	41	6.6	0.65 ± 1.4	38
Ovulatory	18	14	2.9 ± 47	54
Luteal	39	19.8	8.6 ± 1.22	33

* Women from each group with available serum and vaginal smear for analysis.

** day of intravaginal challenge and testing during the menstrual cycle.

† normal ranges for menstrual cycle: Follicular (1–3 ng ml⁻¹); Ovulatory (2–3 ng ml⁻¹); Luteal (> 8 ng ml⁻¹); SEM, standard error of the mean.

‡ Measure of local estrogen influence; determined by vaginal smear stained with Papanicolaou. Superficial is defined as the end-stage differentiation of epithelial cells after exposure to estrogen. Normal ranges for the menstrual cycle: Follicular (20%); Ovulatory (70%); Luteal (60%).

Results were similar for the 11 subjects tested at each of the three stages of the menstrual cycle (data not shown).

Changes in vaginal-associated cytokines over the menstrual cycle

Though immunomodulators remained relatively unchanged following intravaginal challenge with *Candida* antigen over the menstrual cycle, several differences in cytokines were observed at baseline during the three stages of the menstrual cycle. As shown in Figure 5, the Th1-type cytokines appeared to decline from the follicular to the ovulatory stage and then rebound to a higher level during the luteal phase. On the other hand, the Th2-type cytokines tended to increase during the ovulatory and/or luteal phases (an exception was IL-10, which adhered more to the Th1 cytokine pattern). Interestingly, TGF- β was increased substantially during both the ovulatory and luteal phases compared to the follicular phase. Statistical differences compared to the follicular phase included decreased IL-2 in the ovulatory phase ($P < 0.007$), increased IFN- γ and IL-12 in the luteal phase ($P < 0.047$, $P < 0.0001$, respectively), decreased IL-10 in the ovulatory phase ($P < 0.0001$), and increased IL-4 and TGF- β in the ovulatory and luteal phases ($P < 0.002$ and 0.0001 , respectively). With respect to proinflammatory cytokines (Figure 6), IL-1- α was increased between the follicular and ovulatory phases of the menstrual cycle ($P < 0.0015$) and returned to follicular levels during the luteal phase. IL-6 remained unchanged between the follicular and ovulatory phases, but was reduced in the luteal phase ($P < 0.003$). TNF- α was unchanged throughout the menstrual cycle.

TGF- β in vaginal tissue

Based on the high presence of TGF- β in vaginal secretions and the similarly high levels in murine vaginal tissue [17], we examined non-diseased vaginal cuff tissue from hysterectomy patients for TGF- β . Consistent with

the presence in vaginal lavages, fairly high concentrations of TGF- β were detected in vaginal tissue (mean: 190 pg mg⁻¹ protein, median: 172.2 pg mg⁻¹ protein, range: 76–398 pg mg⁻¹ protein).

Discussion

In an attempt to better understand the local immune dysfunction or deficiency in women with RVVC, we have been conducting clinical designs to identify the normal mucosal host response to *C. albicans* at the vagina in women without a history of RVVC. The strategy has been to intravaginally challenge women expected to have intact protective vaginal immunity with *Candida* antigen and detect responses in vaginal lavages. Accordingly, this study was conducted to confirm or deny results from a previous pilot study that revealed evidence for a Th1-type response following intravaginal challenge with *Candida* skin test antigen [19]. As a means to identify influences of reproductive hormones on local immune reactivity, women were stratified according to the stage of the menstrual cycle and did not include those taking hormonal contraception. Special care was also taken to include only those women with positive systemic responses to *Candida* even though most evidence to date suggests that systemic responsiveness does not play a role at the vaginal mucosal against *C. albicans*. Only one of 94 participants was excluded from the analysis based on these criteria. Unfortunately, using these strict criteria and design stratification, there was no evidence of any significant immune stimulation in response to the intravaginal challenge with the *Candida*, including Th1/Th2 and proinflammatory cytokines, total and *Candida*-specific IgE, histamine, or prostaglandin. This was true for both a dilute and concentrated antigen preparation. The diluted formulation was the equivalent of the amount used in an intradermal skin test, while the concentrated formulation was the equivalent of the amount used in 10 individual skin tests. Using

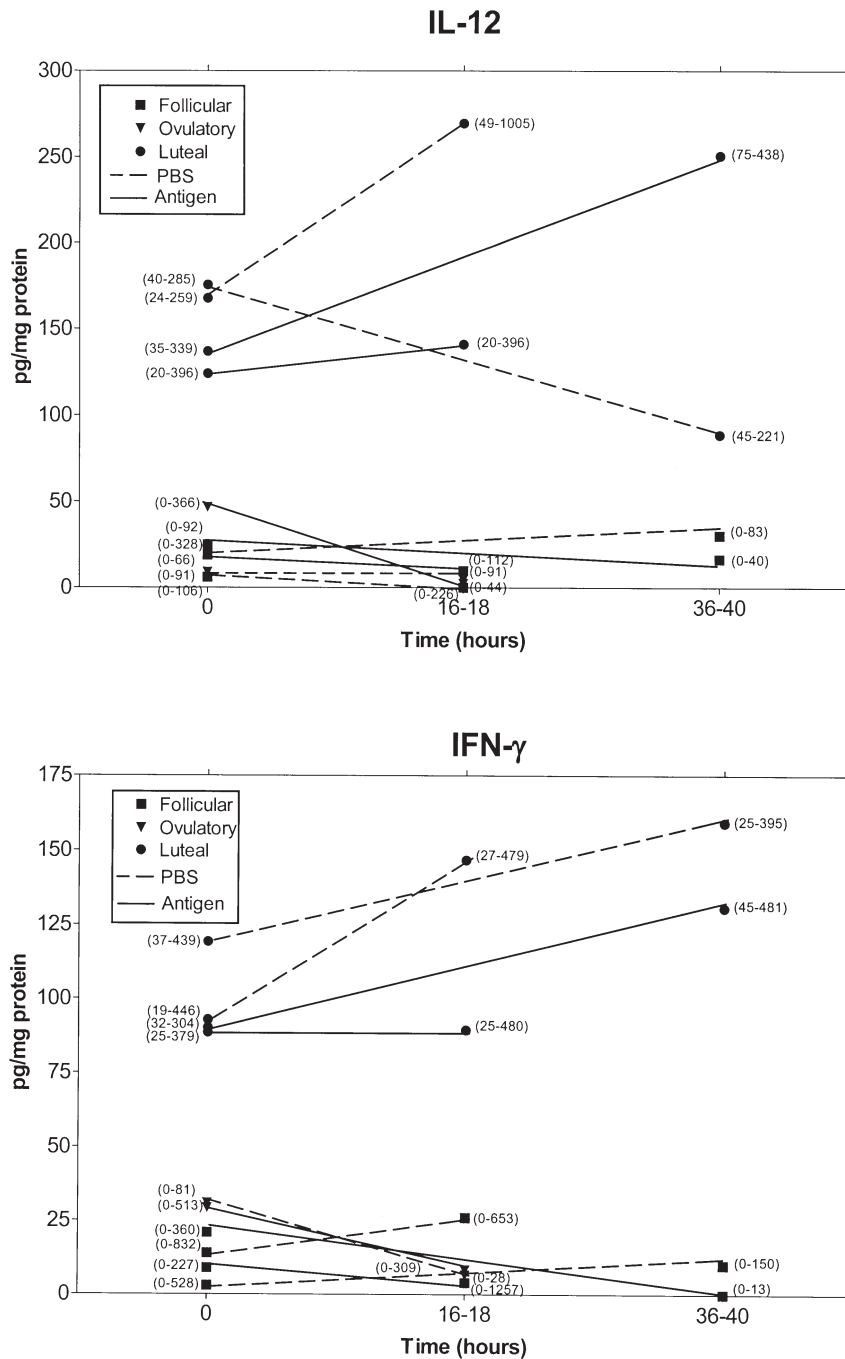


Fig. 1 Local Th1-type cytokines before and after intravaginal challenge with *Candida* antigen. Women ($n = 10-12$ per group) were given a vaginal lavage before and either 16–18 or 36–40 h after intravaginal administration of *Candida* antigen or sterile saline during either the follicular, ovulatory (36–40 h time point was not done), and luteal phase of the menstrual cycle. Th1-type cytokines were evaluated in the lavage fluids by ELISA. Figure shows the median concentrations of IL-12 and IFN- γ normalized to total protein. Ranges are provided in parentheses.

the concentrated antigen, of 13 immunomodulators tested, we observed one change during each of the three stages of the menstrual cycle (IgE-follicular, IL-12-ovulatory, IL-10-luteal). Although each was elevated and antigen-induced, these small numbers of changes in cytokines/immunomodulators do not constitute any pattern that could be considered a substantial immune response or any immunological influence of the reproductive hormones. The lack of changes in immunomo-

dulators, especially proinflammatory cytokines and histamine, was consistent as well with the lack of any evidence of signs and/or symptoms of vaginal inflammation, including erythema, itching, burning, and cellular infiltrates in vaginal lavage fluid. Thus, this study using large numbers of women and properly controlling for the stage of the menstrual cycle with no pharmacological influence of reproductive hormones (i.e., hormone contraceptives) is definitive with the interpretation that

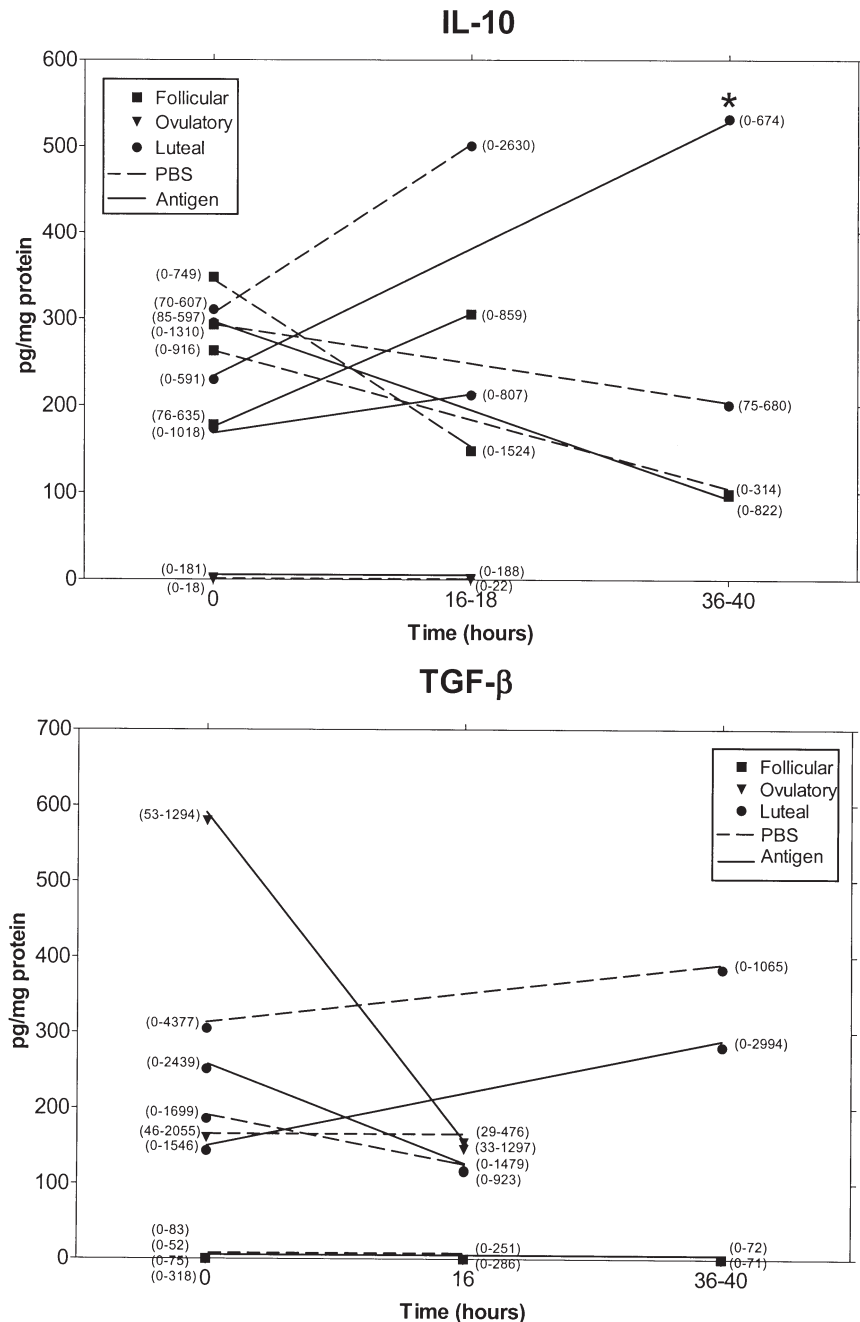


Fig. 2 Local Th2-type cytokines before and after intravaginal challenge with *Candida* antigen. Women ($n = 10-12$ per group) were given a vaginal lavage before and either 16–18 or 36–40 h after intravaginal administration of *Candida* antigen or sterile saline during either the follicular, ovulatory (36–40 h time point was not done), and luteal phase of the menstrual cycle. Th2-type cytokines were evaluated in the lavage fluids by ELISA. Figure shows the median concentrations of IL-10 and TGF- β normalized to total protein. Ranges are provided in parentheses. Asterisk represents statistical significance at $P < 0.05$ compared to time 0.

vaginal immune responses were not stimulated by intravaginal challenge with *Candida* antigen. However, it remains possible that the conditions were still not optimal for adequate immune stimulation, including time post challenge, proper absorption of antigen into the tissue, or antigen concentration. Each seems unlikely though as times consistent with delayed skin test reactivity were employed together with a protocol that was designed to maximize antigen concentration and minimize leakage and promote absorption (small volume given at night when the subject was lying down and less

active). Accordingly, there was no evidence of substantial leakage of the administered contents that could account for a lack of responsiveness. It is also possible that a response was induced, but remained in the submucosa or deeper, precluding any ability to measure evidence of it. If so, however, such responses would be of little benefit to the host in the case of an infection that is superficial. Yet another alternative to the use of lavage fluid to monitor immune responsiveness is to evaluate the presence or absence of lymphoid cells in the lavage for both cell types and associated cytokines. However,

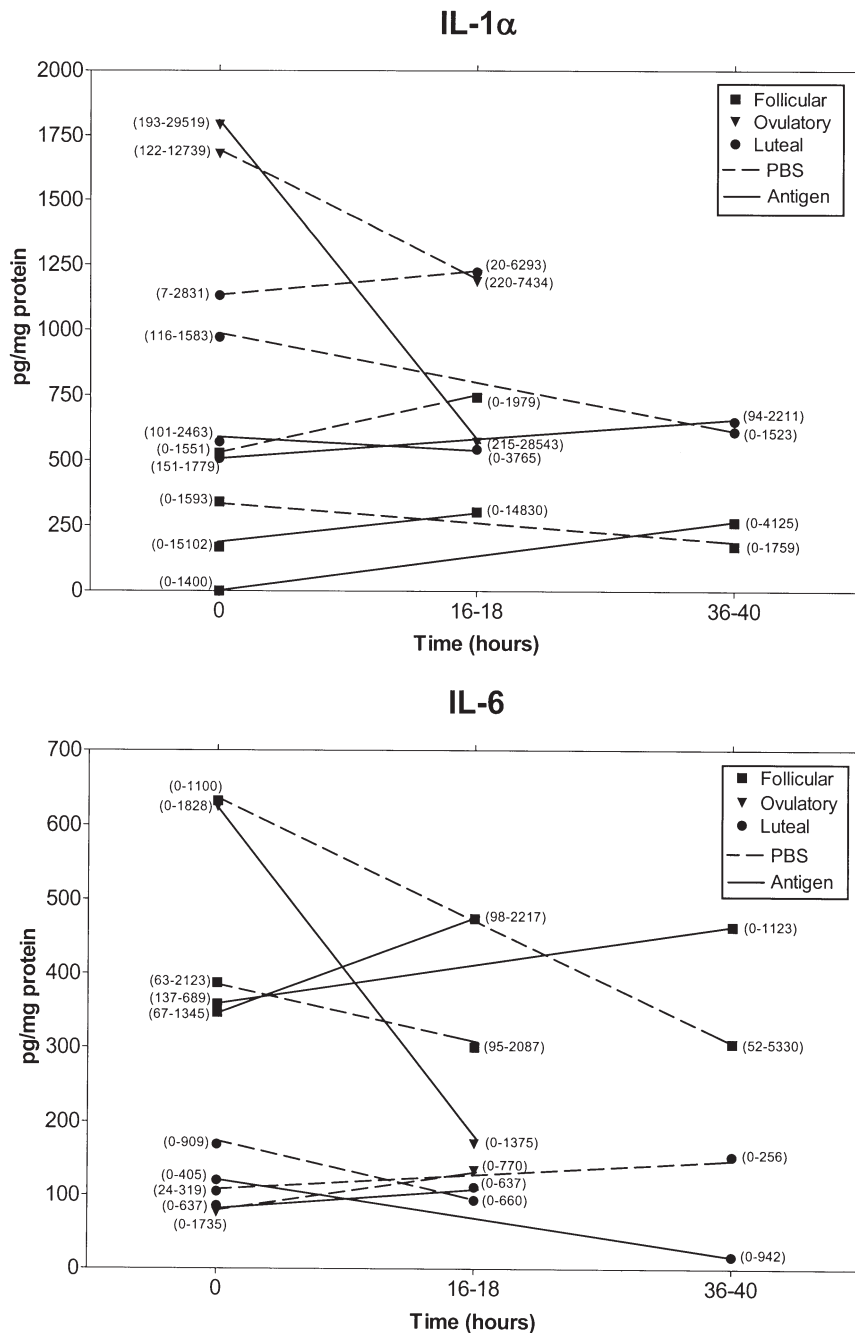


Fig. 3 Local proinflammatory cytokines before and after intravaginal challenge with *Candida* antigen. Women ($n = 10-12$ per group) were given a vaginal lavage before and either 16–18 or 36–40 h after intravaginal administration of *Candida* antigen or sterile saline during either the follicular, ovulatory (36–40 h time point was not done), and luteal phase of the menstrual cycle. Proinflammatory cytokines were evaluated in the lavage fluids by ELISA. Figure shows the median concentrations of IL-1 α and IL-6 normalized to total protein. Ranges are provided in parentheses.

since >98% of the cells collected were epithelial cells (data not shown), the remaining cells were too few in number to identify or evaluate even following enrichment protocols.

If one assumes that the conditions were adequate to stimulate vaginal immune responses, the results are quite unexpected based on the fact that these women are considered protected against bouts of VVC. In fact, 13% of the subjects were asymptotically colonized with yeast at the time of testing with ~70% of the isolates

identified as *C. albicans*. Thus, *Candida* antigen together with live saprotrophic organism did not result in demonstrable responsiveness. On the other hand, these results may not be unexpected taking into account the most recent hypotheses regarding the host response to *C. albicans* as formulated by studies in mice and macaques. In mice, there is now considerable evidence for some form of T cell-mediated immunoregulation or tolerance at the vaginal mucosa against *C. albicans* [16–18]. The presence of high baseline concentrations of TGF- β in the

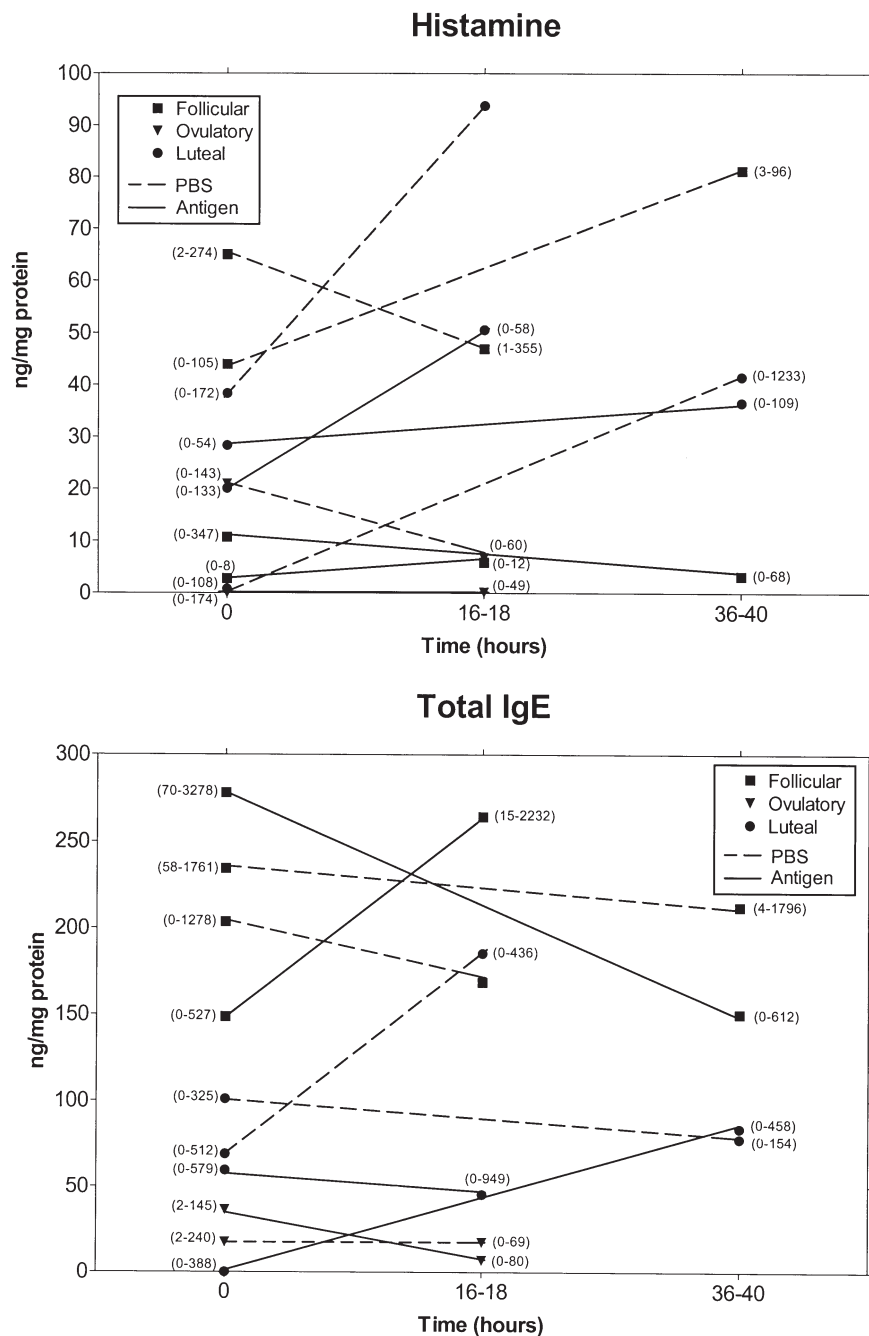


Fig. 4 Local histamine and total IgE before and after intravaginal challenge with *Candida* antigen. Women ($n = 10-12$ per group) were given a vaginal lavage before and either 16–18 or 36–40 h after intravaginal administration of *Candida* antigen or sterile saline during either the follicular, ovulatory (36–40 h time point was not done), and luteal phase of the menstrual cycle. Histamine was quantified by competitive immunoassay while total IgE was quantified by ELISA. Figure shows the median concentrations of each normalized to total protein. Ranges are provided in parentheses.

vaginal lavage fluids and vaginal cuff tissue from the present study provides additional support for this concept although no evidence of elevated TGF- β was evident following antigen challenge as was observed in mice [17]. Additionally, there was no evidence of vaginal changes in PGE $_2$ in the present study as are often seen during conditions of down-regulated immunity. In macaques, an intravaginal challenge with live *C. albicans* to animals asymptotically colonized with yeast species showed evidence of infection (i.e., presence of hyphae

and possible control of the vaginal fungal burden during the observation period 21 days), but still required antifungal intervention to resolve the infection. Interestingly, the macaques did not show any signs or symptoms of infection despite the florid presence of hyphae in the vaginal lumen. Furthermore, there was a decline in the Th2-dominated Th1/Th2 vaginal-associated cytokines during infection with vaginal antibodies remaining stationary [24]. In contrast to these data in humans as well as those from mice and macaques, a rat model of

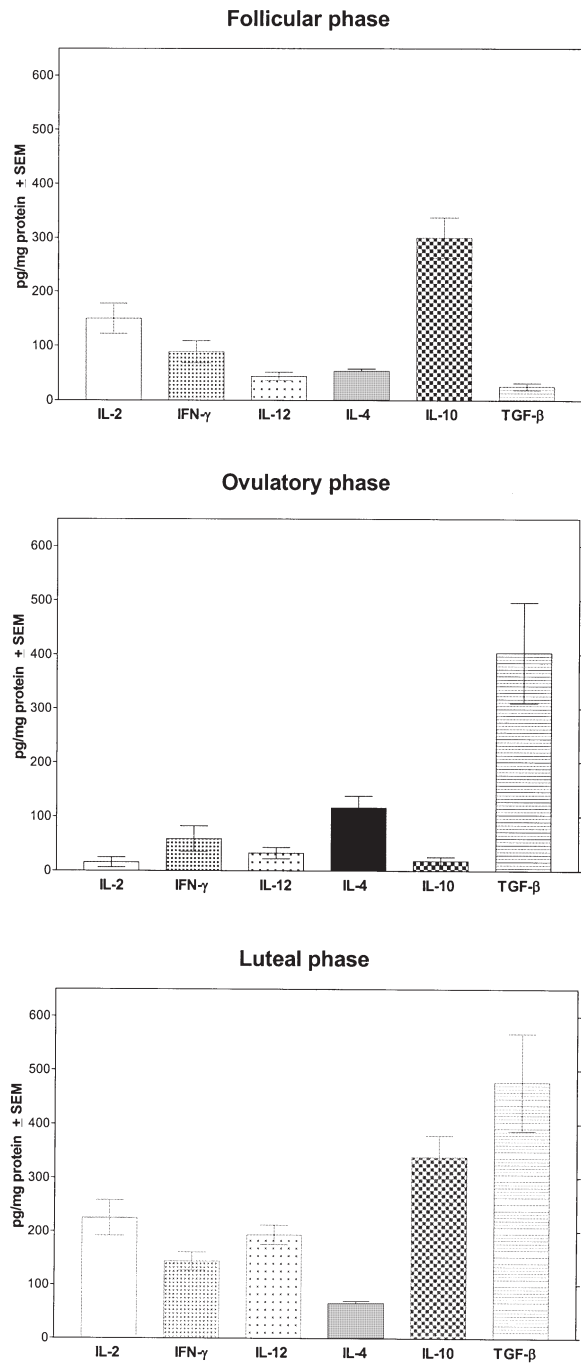


Fig. 5 Th-type cytokines over the menstrual cycle. Baseline concentrations of vaginal-associated cytokines in pre-antigen/saline lavages from the follicular, ovulatory, and luteal phases of the menstrual cycle are illustrated ($n = 10\text{--}12$ for each group). Figure shows mean $\pm$ standard error of the mean (SEM) normalized to total protein.

vaginal candidiasis has provided evidence for protective responses by both antibodies and T cells with considerable T cell infiltration into the vaginal mucosa [25].

Thus, despite intensive clinical studies and the use of

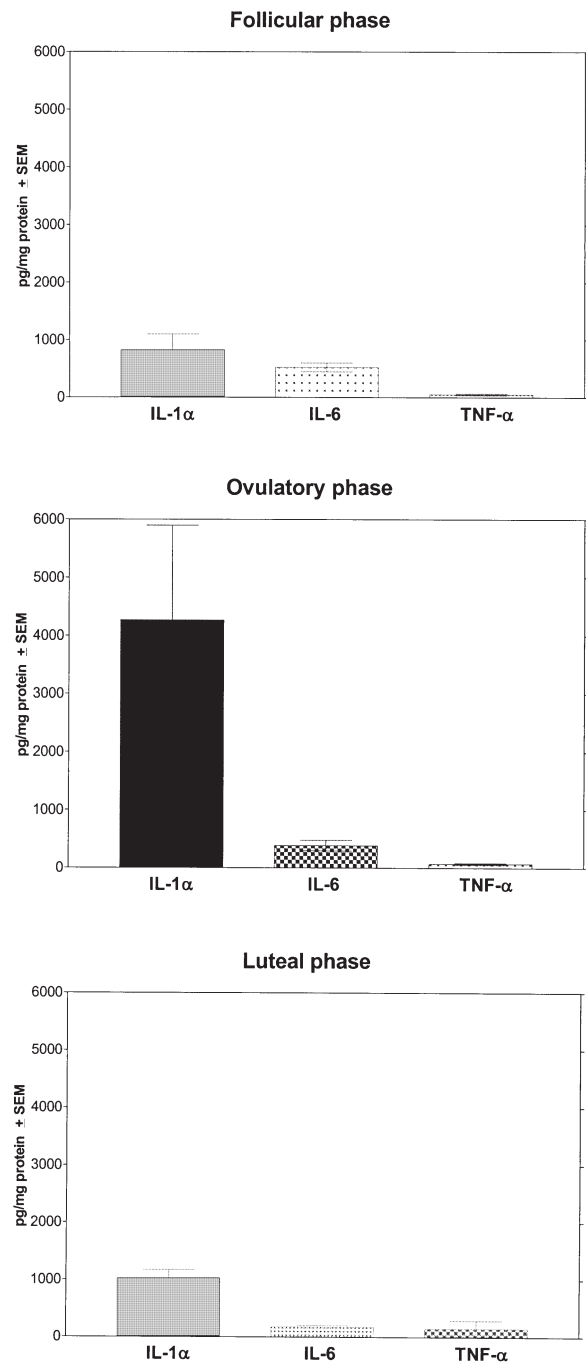


Fig. 6 Proinflammatory cytokines over the menstrual cycle. Baseline concentrations of vaginal-associated cytokines in pre-antigen/saline lavages from the follicular, ovulatory, and luteal phases of the menstrual cycle are illustrated ($n = 10\text{--}12$ for each group). Figure shows mean $\pm$ standard error of the mean (SEM) normalized to total protein.

several animal models, we are currently no closer to identifying the natural protective vaginal host response to *C. albicans* although much evidence points to some form of immunoregulation even though most women

with demonstrable asymptomatic colonization do not become infected. Other possibilities include a level of innate resistance that controls commensalism. Indeed, vaginal epithelial cells have been shown to inhibit the growth of *C. albicans* [26,27]. With the limitations of the clinical design used here as well as those with the animal models, a definitive means to induce or stimulate the protective immune response may require the presence of the live organism. Such live challenge models are currently being used for other pathogens [28,29]. The commensal nature of *Candida*, the superficial nature of the infection, the lack of safety problems when antigen was administered to colonized women, and the ability to successfully treat acute episodes of VVC makes *C. albicans* an excellent candidate for a live challenge model.

Despite the fact that antigen challenge did not modulate cytokines or other immunomodulators at the vaginal mucosa, several changes were observed in cytokines over the course of the menstrual cycle providing clear evidence that such immune mediators modulate under various conditions. Although a mix of Th1 and Th2 cytokines were present at each stage, the general trend was for the Th1 cytokines to become reduced and the Th2 cytokines to become increased between the follicular and ovulatory stages and return to baseline at the luteal phase. The exception was IL-10 that followed the same pattern as the Th1 cytokines and TGF- β that was significantly increased in both the ovulatory and luteal phases. The increases in TGF- β over the menstrual cycle is consistent with the role it may play in immunoregulation or tolerance as discussed earlier. Elevations in TGF- β during the ovulatory and luteal phases of the menstrual cycle are also consistent with the influence of estrogen on TGF- β in mice. Furthermore, bouts of acute VVC are most common during the luteal phase of the menstrual cycle. We would hypothesize based on our data that TGF- β plays a role in susceptibility to VVC by limiting protective responses during those times of the menstrual cycle and may account for the observed hormonal influences of VVC. In the case of the proinflammatory cytokines, fewer changes were observed. The exception was a significant increase in IL-1- α during the ovulatory stage followed by a drop back to follicular levels during the luteal phase. It is unclear why an inflammatory mediator would become elevated at a point in reproduction when the immune response should be suppressed rather than activated. On the other hand, the pleiotropic roles IL-1 has in physiology, metabolism, and general homeostasis may better reflect its role during ovulation.

This study is the second to evaluate vaginal-associated cytokines over the menstrual cycle, although the first

only examined the follicular and luteal phases of the cycle [30]. Nevertheless, similarities include a decrease in IL-6, low TNF- α , and steady concentrations of IL-10 at the follicular and luteal stages of the cycle. In contrast, IL-4 and IFN- γ , undetectable in the previous report, were clearly detected in our study. Interestingly, inclusion of the ovulatory phase in the present study showed several changes that could not have been realized in the previous report. Another issue is the expression of data. The previous study expressed the concentration of cytokines by volume alone. Our study, on the other hand, normalized the concentrations of cytokines to total protein in the lavage fluid. This was probably not a factor in comparing the results of these two studies since the protein content of the individual fluids of the present study was fairly consistent at $\sim 0.6 \text{ mg ml}^{-1}$ and with little modulation during the various stages of the menstrual cycle. Nevertheless, we contend that normalization should be employed to control for any protein variability and allow for easy comparison between studies.

In summary, this study provides evidence that vaginal immune stimulation against *C. albicans* is more difficult than originally reported and may require a significant modification in design to acquire the information necessary to identify the natural host response against *C. albicans* at the human vaginal mucosa. One possibility is a live *Candida* challenge model that would help differentiate between a lack of responsiveness due to technical issues of antigen choice or adequate absorption versus a true immunoregulatory or tolerance mechanisms. Certainly the cytokines are present in vaginal secretions that could influence the local host response and appear to modulate during the menstrual cycle in a manner that could promote acute VVC. But not until the natural host defense mechanisms are understood in direct response to *C. albicans* in healthy women, will our understanding of immunopathogenesis in RVVC be realized and immunotherapeutic strategies to prevent or treat the infection be attainable.

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