Vaginal-Associated Immunity in Women with Recurrent Vulvovaginal Candidiasis: Evidence for Vaginal Th1-Type Responses following Intravaginal Challenge with Candida Antigen

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Studies from women with recurrent vulvovaginal candidiasis (RVVC) and from an animal model of experimental vaginitis suggest that deficiencies in immune function should be examined at the local rather than systemic level. Evidence of vaginal cell-mediated immunity (CMI) was evaluated for the first time in cervicovaginal lavage (CVL) fluid from RVVC patients. Results showed that although constitutive Th1- and Th2-type cytokine expression was detectable in CVL fluid from normal women, and differences in cytokines were observed in RVVC patients, limitations in experimental design of such de novo analyses urged caution in interpretation. Alternatively, attempts were made to establish conditions in control subjects whereby vaginal immunity could be detected after intravaginal challenge with Candida antigen. Preliminary results showed that Th1-type cytokines (interleukin-2 and -12, interferon-γ) and histamine were increased 16–18 h after intravaginal introduction of Candida skin test antigen. Intravaginal antigenic challenge represents a novel approach for studying Candida-specific vaginal CMI.

Recurrent vulvovaginal candidiasis (RVVC) is an opportunistic mucosal infection caused by Candida albicans that affects up to 5% of otherwise healthy women of child-bearing age [1]. Although antifungal resistance does not appear to play a major role in recurrence [2], women with RVVC usually experience repeat episodes shortly after successful therapeutic intervention [1]. While several exogenous factors (antibiotics and oral contraceptives) predispose some women to acute VVC [1], the underlying factors contributing to RVVC are largely unknown. Because of the high incidence of mucosal candidiasis in patients with reduced cell-mediated immunity (CMI), such as those with AIDS [3, 4], transplantation [5], and corticosteroid therapy [6], it is postulated that deficiencies in CMI also play a significant role in the etiology of RVVC.

Studies examining systemic CMI in peripheral blood of women with RVVC have thus far failed to show a deficiency or distinct pattern of reduced activity [7–9], although reduced Candida-specific skin test reactivity and peripheral blood lymphocyte (PBL) proliferation have been reported [7, 8, 10–12]. Similarly, comprehensive studies involving extensive cytokine analyses of RVVC patients during both symptomatic episodes and asymptomatic periods of remission, including longitudinal analyses, showed that PBL from women with RVVC had normal Th1-type cytokine expression in response to Candida antigens [8], which is characteristic of protective immune reactivity to C. albicans [13, 14].

Results from an estrogen-dependent animal model of experimental vaginal candidiasis have paralleled these clinical observations. Results indicate that although a localized vaginal C. albicans infection stimulates Candida-specific systemic Th1-type CMI [15, 16], preinduced Candida-specific systemic CMI was not protective against vaginal candidiasis [17]. In contrast, however, mice given a spontaneously resolving primary vaginal infection in the absence of estrogen were partially protected from a second vaginal infection under pseudoestrus conditions [18]. In these experiments, inhibition of Candida-specific systemic CMI had no effect on this protection, suggesting the presence of a locally acquired mucosal immune response [18]. Similarly, the depletion of systemic CD4 and CD8 T cells with neutralizing antibodies did not affect the natural history of primary or secondary vaginal infections [19]. Taken together, our data suggested that local is more important than systemic CMI for protection against vaginal C. albicans infection.

To date, local vaginal immunity in women with RVVC has been evaluated only by measurement of immunoglobulin levels in vaginal secretions. Interestingly, while Candida-specific IgA was shown to be normal in women with RVVC [20], Candida-specific IgE was often elevated [21–23], indicating a potential for Th2-type cytokine expression and the presence of immedi-
ate hypersensitivity. Although a paucity of information exists for CMI at the cervicovaginal level, the vaginal mucosa appears to contain all of the necessary components to elicit competent CMI, including class II major histocompatibility complex cells and T lymphocytes [24, 25]. In fact, these cells may be uniquely compartmentalized, as we [26] and others [27, 28] have shown that murine vaginal T cells are phenotypically distinct from those in the periphery.

The purpose of this study was to assess local vaginal immune reactivity in women with RVVC by cytokine expression in cervicovaginal lavage (CVL) fluid collected during symptomatic infection and during periods of infection-free remission. The results were compared with cytokines produced by the patient PBL in response to Candida antigens in vitro and to local and PBL-mediated cytokines in normal healthy women without a history of vaginal candidiasis.

Materials and Methods

Subjects in RVVC Study

Women with RVVC (n = 24) were 21–50 years of age, otherwise healthy, and nonpregnant and had had at least three documented episodes of RVVC during the previous 12 months, evidenced by signs or symptoms, positive KOH smear, and positive vaginal culture for C. albicans. Eligible patients were enrolled through the Mott Center Vaginitis Clinic at Wayne State University School of Medicine. Patients were further classified at testing into the culture-positive RVVC group (n = 16), defined as those with a symptomatic episode of recurrent C. albicans vaginitis, and the culture-negative RVVC group (n = 18), defined as RVVC patients who were asymptomatic with a negative vaginal culture at the time of evaluation. Ten of the culture-positive RVVC patients were tested longitudinally, during a symptomatic episode and again when culture-negative. The control group consisted of healthy asymptomatic nonpregnant women between the ages of 21 and 50 with no history of vaginal candidiasis (n = 16).

Specimens Collected

Blood (20–40 mL) was collected by venipuncture into heparinized tubes, a vaginal swab was collected, and a CVL was performed with 5 mL of nonpyrogenic sterile saline. The CVL fluid was collected after 30–40 s of aspiration. No evidence of blood was present in any CVL fluid, reducing the possibility that the fluid was contaminated with systemically derived immune molecules. The vaginal swab was streaked onto Sabouraud dextrose agar plates and incubated at 30°C for 48 h. C. albicans–derived colonies were confirmed by germ tube formation in fetal bovine serum. The CVL fluid was clarified and the fluid was collected, filtered through a 0.22-μm membrane, aliquoted in 0.5-mL aliquots, and frozen at −70°C until use. Total protein content in each CVL fluid sample was determined by the Lowry method with a commercial kit (Sigma, St. Louis). PBL were isolated from the blood by use of ficoll-hypaque density gradient centrifugation as previously described [8].

Systemic Immune Reactivity

PBL from each patient and control subject were cultured in vitro for lymphoproliferation and cytokine production in response to a mitogen, soluble and particulate Candida antigens, and a bacterial antigen.

Antigens and mitogens. The mitogen was phytohemagglutinin (PHA; Sigma). Two C. albicans antigens were used. C. albicans heat-killed blastospores (HKB) were prepared by incubating a fresh stationary-phase blastospore 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 substance (SCS) was a gift of Judith Domer (Tulane University School of Medicine, New Orleans). Tetanus toxoid (Lederle Laboratories, Pearl River, NY) served as the bacterial antigen.

Proliferation assays. Proliferation assays were used as a general measure of lymphocyte activity to Candida antigens. PHA was included as a quality control for positive lymphocyte responses. The methods used in our laboratory for proliferation assays with PBL have been described previously [8]. Briefly, 1.5 × 10⁶ PBL/mL were cultured in triplicate in sterile 96-well tissue culture plates (Costar, Cambridge, MA) in a total volume of 200 μL. Cells were incubated at 37°C with 5% CO₂ with serum-free AIM V lymphocyte medium (Life Technologies GIBCO BRL, Gaithersburg, MD) supplemented with 100 mM glutamine or with AIM V/glutamine medium containing 5 × 10⁶ C. albicans HKB, 125 μg/mL C. albicans SCS, or 20 μg/mL PHA. On the third (mitogen) or sixth (antigens) day of culture, 1.0 μCi of [³H]thymidine (ICN Radiochemicals, Irvine, 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 cells was counted in a liquid scintillation counter (Beckman Instruments, Irvine, CA). Data were expressed as proliferation indices (mean counts for stimulated cultures/mean counts for unstimulated cultures).

Cytokine production. Cytokine production was assessed by the culture of 3 × 10⁶ PBL/mL incubated in 24-well tissue culture plates at 37°C with 5% CO₂ with medium alone (AIM V/glutamine) or additionally with 20 μg/mL PHA, 5 × 10⁶ HKB/mL, 125 μg/mL SCS, or tetanus toxoid diluted 1:5 in a volume of 2 mL. The 48-h supernatants were collected, clarified, and stored at −70°C until use.

Immunologic Assays

The tissue culture supernatants and CVL fluid were assessed for immunoglobulins and Th1- and Th2-type cytokine expression. The Th1-type cytokines included interleukin (IL)-2, interferon (IFN)-γ, and IL-12. The Th2-type cytokines included IL-4, IL-5, and IL-10. CVL fluid was also evaluated for immunoglobulins, including total and Candida–specific IgG, IgA, and IgE. Immune mediators in culture supernatants were expressed as specific units of cytokine or immunoglobulin per milliliter (concentration in antigen-stimulated cultures minus that in unstimulated cultures), while mediators in CVL fluid were expressed as units of cytokine or immunoglobulin per milligram or microgram of protein.

IL-12 and IFN-γ. IL-12 and IFN-γ in the culture supernatants or lavage fluid were quantitated by a sensitive and specific com-
mmercial ELISA (R&D Systems, Minneapolis; Genzyme, Cambridge, MA). The standard curve for each assay was generated in saline or AIM/glutamine medium to obtain the identical matrix of the lavage fluid and culture supernatants, respectively. The CVL fluids or supernatants were assayed in duplicate using 50–100 μL well. The quantitation of each cytokine in picograms of cytokine per milliliter (culture supernatants) or picograms of cytokine per milligram of protein (lavage fluid) was analyzed by use of an automated microplate reader (Ceres 900; Bio-Tek, Winooski, VT) and Kineticalc software (Bio-Tek).

**IL-2, IL-4, IL-5, and IL-10.** IL-2, IL-4, IL-5, and IL-10 in the CVL fluids were quantitated by ELISA using commercial capture and biotinylated detection antibodies (PharMinGen, San Diego) and the respective recombinant human cytokine for generation of the standard curve. The assays were performed in high protein binding EIA/A2 96-well plates (Costar) according to manufacturer’s instructions. Briefly, the plates were coated with the capture antibody (1 μg/mL) in coating buffer (0.1 M NaHCO₃) and incubated overnight at 4°C. The plates were washed four times with PBS + 1% bovine serum albumin (BSA) + 0.05% Tween 80 (wash buffer). The nonspecific binding sites were blocked with PBS + 3% BSA + 0.05% Tween 80 (blocking buffer) for 3 h at room temperature. After the plate was washed several times, the standard and unknown samples were added to each well in duplicate using a volume of 50 μL and incubated for 2 h at room temperature. After this incubation, the plate was washed again several times, and the biotinylated detection antibody (1 μg/mL) was added to each well and incubated for 1 h at room temperature. After several additional washings, a solution of avidin–alkaline phosphatase (diluted 1:50,000) was added, followed by addition of the substrate p-nitrophenyl phosphate (Sigma). Quantitation of cytokines in CVL fluid was done by use of the Ceres 900 plate reader at 405 nm as described above.

**Total IgG, IgA, and IgE.** A sandwich ELISA was done in 96-well EIA/A2 plates. Briefly, anti-human IgG (1 μg/mL), IgA (3 μg/mL), or IgE (10 μg/mL) antibodies (capture antibody) (Dako) diluted in coating buffer (0.1 M NaHCO₃) was added to each well in a volume of 50 μL and incubated overnight at 4°C. On the following day, the plate was washed four times with wash buffer (PBS + 1% BSA + 0.05% Tween 80), and nonspecific binding sites were blocked with 100 μL/well blocking buffer (PBS + 3% BSA + 0.05% Tween 80) for 2 h at room temperature. After the plate was washed four times, samples were added in a volume of 50 μL/well and incubated for 3 h at room temperature. Preliminary studies revealed that CVL fluid diluted 1:20 was optimal for detecting IgA and IgG, whereas undiluted samples were required for IgE determinations. Human IgG and IgA (Sigma) were used to generate standard curves for the quantitation of IgG and IgA. IgG was serially diluted (2-fold) from 125 to 2 ng/mL. IgA was serially diluted (2-fold) from 1000 to 15 ng/mL. Since there was no commercially available human IgE at the time of this study to use as the standard, we used a pooled sample of all CVL fluids that showed positive absorbances (at least 2-fold above the mean absorbance of saline alone). The sample was serially diluted (2-fold) from 1000 to 15 U/mL in sterile saline and added in a volume of 50 μL to separate wells. The blank wells contained 50 μL of sterile saline. At the conclusion of the incubation period, the wells were washed four times, and alkaline phosphatase–conjugated anti-human IgG (1:10,000), IgA (1:20,000), or IgE 1:1000 (all from Sigma) was added to each well in a volume of 50 μL and incubated for 1 h at room temperature. After washing four times, 50 μL of the p-nitrophenyl phosphate substrate (Sigma) was added to each well, and the reaction was allowed to develop for 20–60 min. The reaction was stopped with the addition of 50 μL of 3 N NaOH. Quantitation of each antibody was analyzed by use of the Ceres 900 microplate reader and Kineticalc software as described above. Results for IgG and IgA were expressed as nanograms of immunoglobulin per microgram of protein, while results for IgE were expressed as units per milligram of protein.

**Candida-specific IgG, IgA, and IgE.** Candida-specific immunoglobulin was also quantitated by ELISA by use of the same basic methodology as described above with the exception of the capture procedure and the standard. In this assay, instead of a capture antibody, *C. albicans* culture filtrate antigen (CaCF) [15] diluted to 10 μg/mL in coating buffer was used to coat the plates. Since there is no commercially available Candida-specific IgG, IgA, or IgE to use as a standard, we used an approach similar to that described above for total IgE by use of a pooled sample of lavage fluids having absorbances above that of sterile saline. This pooled sample was derived predominantly from RVVC patients. The sample was diluted from 1000 to 15 U/mL in sterile saline. Preliminary studies showed that a 1:20 dilution of lavage fluid was optimal for IgG determinations, while undiluted samples were required for IgA and IgE determinations. All other aspects were identical to the detection of total immunoglobulin, with results expressed as units of immunoglobulin per milligram of protein.

**Subjects Enrolled for Stimulation of Vaginal Anti-Candida Immunity**

Healthy nonpregnant women between the ages of 21 and 50 without a history of RVVC were recruited. A total of 70 were enrolled. All participants were asked to abstain from sexual intercourse during the testing period. Specific data on the subjects enrolled were as follows: 22 (31%) were taking oral contraceptives, 6 (9%) were menopausal, with 2 receiving hormone replacement therapy, and 2 (3%) had had hysterectomies. About 25% of the subjects were taking minor medication or antihistamines. Thirty percent of the subjects were minorities. Each subject was tested during the follicular stage of the menstrual cycle (within 2 days after the end of menses), when estradiol and progesterone are minimal.

**Clinical protocol.** Subjects were stratified into three clinical protocols. The protocols and analyses were conducted at Wayne State University School of Medicine and Louisiana State University Medical Center. In each protocol, 20 mL of blood was drawn from each participant, and a vaginal swab and CVL fluid were collected as described above. For protocol A (n = 30), each participant was given a vaginal suppository (gelatin capsule) containing 10⁶ *C. albicans* HKB or sterile PBS alone. For protocol B (n = 24), each participant was given a syringe kit prepared by a research nurse (D. Leaman) containing 10⁶ *C. albicans* HKB in 1.0 mL of PBS or PBS alone. For protocol C (n = 16), the participant was given the syringe kit containing soluble *Candida* skin test antigen (Berkeley Biologicals, Berkeley, CA) diluted 1:10 in PBS or PBS alone. The commercial Candida antigen contained no adjuvants or other mediators of nonspecific immune reactivity. In each proto-
col, the participant was instructed to administer the suppository or 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. This evening protocol was used to avoid leakage. For each protocol, the participant was blinded to the contents inserted. For all protocols, the participant was instructed to return to the clinic or office at a specified time for a second collection of blood (for serum only) and CVL fluid. In protocol A, subjects were instructed to return either 72 h \( (n = 15) \) or 120 h \( (n = 15) \) after antigen or saline administration. In protocol B, subjects were instructed to return either 9–11 h \( (n = 8) \), 18–20 h \( (n = 8) \), or 30–33 h \( (n = 8) \) after antigen or saline administration. In protocol C, the subjects were instructed to return either 16–18 h \( (n = 8) \) or 36–40 h \( (n = 8) \) after antigen or saline administration. The rationale regarding timing of the second CVL was to evaluate short and long time periods in the case of particulate antigens that may require more time for absorption and a relatively short time for soluble antigens that absorb more readily.

The initial vaginal swab was used to identify vaginal colonization by \textit{C. alibicans}. The blood was used for the isolation of serum and PBL (initial draw only) as described above. The CVL fluid was prepared as described above. The serum sample was used for estradiol measurements to verify that subjects were tested during the follicular stage of the menstrual cycle. PBL were used in the proliferation study described above with PHA and \textit{C. albicans} HKB and SCS to verify systemic sensitization to \textit{Candida} antigens. The CVL fluid was used to quantitate the cytokines and immunoglobulins as described above. The CVL fluid was also evaluated for histamine content as an additional indicator of immediate hypersensitivity. Comparisons were made between initial and final lavages. Results were expressed as median responses as a function of time between antigen introduction and the second CVL.

Cytokine and immunoglobulin determinations. These assays were done in a manner identical to that described above; PBS was used as the matrix for the standards.

Histamine. Histamine in CVL fluid was quantitated by a competitive EIA kit (Immunootech International, Westbrook, ME). To an antihistamine antibody–coated microtiter plate, an acetylcholinesterase-histamine conjugate was added to each well together with and IgG were not different between controls and symptomatic RVVC patients compared with controls \( (P < .0005) \). Figure 2 illustrates the local expression of Th2-type cytokines (IL-4, IL-5, IL-10) for controls and patients. Although Th2-type cytokines were also detected in CVL fluid collected from control subjects, fewer numbers of subjects showed detectable levels than showed detectable levels of Th1-type cytokines. While median quantities of IL-2 and IFN-\( \gamma \) were not different between controls and RVVC patients, IL-12 in CVL fluid of RVVC patients was significantly reduced compared with that in control subjects \( (P < .0005) \).

Evaluation of immunoglobulin levels in CVL fluid (table 1) showed that although total IgA and IgG were reduced in symptomatic and asymptomatic RVVC patients compared with controls \( (P < .038 \) and .009, and \( P < .02 \) and .003, respectively), similar to previous reports [20], \textit{Candida}-specific IgA and IgG were not different between controls and symptomatic or asymptomatic RVVC patients. While total IgE was low in all groups of subjects, with no significant differences observed, similar to previous studies [21–23], \textit{Candida}-specific IgE was significantly elevated in symptomatic RVVC patients compared with controls or asymptomatic RVVC patients \( (P < .001) \).

The results of cytokine expression in the CVL fluid were compared with cytokine production by PBL in response to the mitogen PHA, \textit{Candida} antigens, and the bacterial antigen tetanus toxoid. As reported in a previous study from our laboratory [8], Th1-type cytokine production (IL-2 and IFN-\( \gamma \)) in response to PHA, soluble (SCS) and particulate (HKB) \textit{C. alibicans} antigens, and tetanus toxoid was similar between controls and symptomatic and asymptomatic RVVC patients (data not shown). In response to \textit{C. alibicans} HKB, however, IL-12 production by PBL (not previously evaluated) was reduced in symptomatic RVVC patients compared with controls (median, 17.2 pg/mL; range, 0–490; vs. median, 99 pg/
Figure 1. Vaginal-associated Th1-type cytokine (interleukin [IL]-2, IL-12, interferon [IFN]-γ) expression in women with RVVC. RVVC patients were tested during symptomatic infection (RVVC⁺) or during period of asymptomatic infection-free remission (RVVC⁻). Controls were healthy women with no history of vaginal candidiasis. Cytokines were evaluated in vaginal lavage fluid. * Significant difference (P < .05) compared with controls.

mL; range, 0–1041; P < .005), but not in asymptomatic RVVC patients (median, 165 pg/mL; range, 0–671). No differences were observed between controls or RVVC patient PBL in response to C. albicans SCS, PHA, or tetanus toxoid (data not shown).

Figure 2. Vaginal-associated Th2-type cytokine (interleukin [IL]-4, -5, and -10) reactivity in women with RVVC. RVVC patients were tested during symptomatic infection (RVVC⁺) or during periods of asymptomatic infection-free remission (RVVC⁻). Controls were healthy women with no history of vaginal candidiasis. Cytokines were evaluated in vaginal lavage fluid. * Significant difference (P < .05) compared with controls.

Systemic Th2-type cytokine expression had not been previously evaluated in PBL cultures for RVVC patients. Results in this study showed that while significant differences were observed, the differences were not specific to Candida anti-
IL-4 was significantly increased in asymptomatic RVVC patient PBL cultures compared with controls and symptomatic RVVC patients in response to HKB (median, 55 pg/mL; range, 0–252; vs. median, 20 pg/mL; range, 0–57; P < .01; or median, 3 pg/mL; range, 0–12; P < .002, respectively), SCS (median, 43 pg/mL; range, 0–230; vs. median, 14 pg/mL; median, 0–182; P < .003; or median, 10 pg/mL; range, 0–90; P < .02, respectively), and tetanus toxoid (median, 56 pg/mL; range, 0–723; vs. median, 0 pg/mL; range, 0–136; P < .0005; or median, 0 pg/mL; range, 0–76; P < .0006, respectively). IL-5 was significantly decreased in symptomatic and asymptomatic RVVC patient PBL cultures compared with controls in response to HKB (median, 2.4 pg/mL; range, 0–64; and median, 21 pg/mL; range, 0–508; vs. median, 67 pg/mL; range, 0–1409; P < .004 and .03, respectively), SCS (median, 6 pg/mL; range, 0–88; and median, 58 pg/mL; range, 0–732; vs. median, 397 pg/mL; range, 0–1363; P < .005 and .04, respectively), and tetanus toxoid (median, 15 pg/mL; range, 0–128; and median, 0 pg/mL; range, 0–211; vs. median, 90 pg/mL; range, 0–340; P < .013 and .026, respectively). Finally, IL-10 was significantly increased in asymptomatic RVVC patient PBL cultures compared with controls and symptomatic RVVC patients in response to PHA (median, 1518 pg/mL; range, 78–3801; vs. median, 581 pg/mL; range, 0–2000; P < .005; and median, 664 pg/mL; range, 0–2478; P < .004), decreased in asymptomatic RVVC patient cultures compared with controls for responses to SCS (median, 0 pg/mL; range, 0–347; vs. median, 460 pg/mL; range, 0–2000; P < .004), and decreased in both symptomatic and asymptomatic RVVC patients compared with controls in response to tetanus toxoid (median, 38 pg/mL; range, 0–300; and median, 45 pg/mL; range, 0–770; vs. median, 395 pg/mL; range, 0–7834; P < .004 and .01, respectively).

### Stimulation of vaginal immunity with Candida antigen

To circumvent potential problems associated with dynamics and antigen specificity in de novo analyses of cervicovaginal secretions from RVVC patients, we examined the potential to stimulate local vaginal immune reactivity in vivo in normal healthy women without a history of vaginal candidiasis. We reasoned that since most healthy persons are sensitized to *C. albicans*, it may be possible to stimulate a local immune response in women by the intravaginal introduction of *C. albicans* antigen. Several protocols were tested covering several time intervals for immune stimulation and different *Candida* antigen preparations. Each protocol involved two CVLs for the detection of

### Table 2. Immunoglobulin expression in vaginal lavage fluid from RVVC patients.

<table>
<thead>
<tr>
<th>Immunoglobulin</th>
<th>Symptomatic RVVC (n = 16)</th>
<th>Asymptomatic RVVC (n = 18)</th>
<th>Control (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgA*</td>
<td>0.9 (0.2–4.1)</td>
<td>0.8 (0.1–2.3)</td>
<td>2.9 (0–17.8)</td>
</tr>
<tr>
<td>Candida-specific IgA'</td>
<td>104 (0–11,000)</td>
<td>453 (0–2500)</td>
<td>1233 (0–26,000)</td>
</tr>
<tr>
<td>IgG*</td>
<td>0.9 (0.2–12)</td>
<td>0.6 (0–2)</td>
<td>4.9 (0–31)</td>
</tr>
<tr>
<td>Candida-specific IgG'</td>
<td>762 (0–3900)</td>
<td>888 (0–3800)</td>
<td>1061 (200–14,400)</td>
</tr>
<tr>
<td>IgE'</td>
<td>0 (0–83)</td>
<td>0 (0–10)</td>
<td>0 (0–180)</td>
</tr>
<tr>
<td>Candida-specific IgE'</td>
<td>20 (0–76)</td>
<td>0 (0–76)</td>
<td>0 (0–3000)</td>
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</tbody>
</table>

*Ng/µg of protein.

† Units/mg of protein.
vaginal immunity, one before antigen administration and one at a specified time after antigen administration. All participants were tested during the follicular phase of the menstrual cycle (within 2 days after the end of menses) to avoid influences of reproductive hormones. Concentrations of cytokines and antibodies were expressed per milligram of total protein in CVL fluid. Results were evaluated by comparing immune mediators in the initial CVL sample to those in the CVL sample after antigen administration.

Protocols A and B involved the intravaginal introduction of $10^8$ *C. albicans* HKB in either a suppository or syringe kit, respectively. Participants ($n = 54$) were stratified into groups that received antigen ($n = 35$) or saline alone (control) ($n = 30$) and by time of vaginal lavage after introduction of the antigen or saline (72 or 120 h for protocol A, $n = 30$; and 9–11, 18–20, 30–33 h for protocol B, $n = 24$). Estradiol measurements in sera showed that all subjects were tested under protein. Constitutive expression of each immunoglobulin was, however, observed (total IgA, 100–600 ng/mg of protein; total IgG, 100–240 ng/mg of protein; *Candida*-specific IgA, 350–700 U/mg of protein; *Candida*-specific IgG, 600–1500 U/mg of protein).

Total and *Candida*-specific IgE as well as histamine content in the CVL fluids is illustrated in figure 5. Constitutive expression of each immune mediator was observed before intravaginal administration of PBS or *Candida* antigen. However, compared with that at time 0 for each respective group, neither total nor *Candida*-specific IgE was increased in CVL fluid 16–18 or 36–40 h after intravaginal administration of *Candida* antigen or PBS. In contrast, histamine was significantly increased in CVL fluid collected 16–18 h after challenge with *Candida* antigen ($P < .048$) compared with that at time 0, but not in subjects given PBS or in those subjects tested 36–40 h after administration of *Candida* antigen or PBS.

**Discussion**

This clinical study examined the vaginal presence of humoral immunity and CMI in women with RVVC and for the first time evaluated Th1- and Th2-type cytokines in CVL fluid and assessed the potential to stimulate putative vaginal-associated immunity in healthy women by intravaginal challenge with *Candida* antigen. The presence of Th1- or Th2-type cytokines in CVL fluid from RVVC patients or from normal healthy women before any exogenous antigen stimulation is the first evidence of its kind suggesting that these cytokines, like immunoglobulins, are constitutively produced in vaginal secretions. Our data suggest that constitutive Th1-type cytokines in the vagina predominate over Th2-type cytokines. The presence of Th1- and Th2-type cytokines in CVL fluid in normal healthy women is consistent with the recent report of the presence of proinflammatory cytokines (tumor necrosis factor, IL-1, and IL-6) in CVL fluid in both human immunodeficiency virus–infected and noninfected women [29]. On the basis of considerable evidence in both clinical studies and experimental animal models suggesting a limited role for systemic immunity at the vaginal mucosa [7, 8, 17–19], it would seem unlikely that the constitutive cytokine production in CVL fluid was contaminated by systemically
derived cytokines. Furthermore, there was no evidence of blood contamination in CVL fluid. The more likely explanation for the constitutive cytokine production is some level of immune reactivity to normal bacterial or fungal flora.

Results for RVVC patients showed that while the majority of Th1- and Th2-type cytokines in CVL fluid were unchanged in RVVC patients compared with controls, significant reductions in both IL-12 (Th1-type) and IL-5 (Th2-type) were observed during episodes of symptomatic vaginitis as well as during periods of remission. Longitudinal assessment of the 10 RVVC patients tested during symptomatic and asymptomatic periods supports cumulative data suggesting that the absence of or decrease in these two cytokines continued in the asymptomatic periods of remission. A decrease in or absence of IL-12 could have important clinical implications in RVVC. IL-12 is an important regulatory cytokine that enhances Th1-type responses and inhibits Th2-type responses [30], influences IFN-γ production [31], and enhances NK cell activity [32]. Although levels of IFN-γ were low in most controls and RVVC patients, reduced levels of IL-12 in RVVC patients may hinder their ability to produce IFN-γ during infection, which may ultimately affect both resistance-associated Th1-type reactivity [13, 14, 33–35] and the induction and recruitment of NK cells into the vaginal mucosa. Similarly, low levels of IL-5 in RVVC patients would suggest a lower incidence of eosinophilia [36] in RVVC, although this contradicts a previous report showing the presence of eosinophils in vaginal smears collected from women with RVVC [22].

Systemic PBL responses from control subjects to mitogens and Candida antigens showed a mix of Th1- and Th2-type cytokines indicative of Th0-type reactivity, while responses to tetanus toxoid were dominated by Th2-type cytokines. Similar to results of our previous study [8], Th1-type cytokines elaborated in response to Candida antigen by patient PBL were not different from controls in the majority of subjects, suggesting again that women with RVVC have normal Th1-type systemic Candida-specific CMI. The only exception was a transient decrease in IL-12 (not previously examined) in PBL from symptomatic RVVC patients stimulated with one of the two Candida antigens. Although our past studies had not assessed Th2-type cytokines in RVVC PBL cultures, the results of the present study showed both in-

![Graph of IL-2, IL-12, and IFN-γ concentrations over time](image)

**Figure 3.** Th1-type cytokine (interleukin [IL]-2, IL-12, interferon [IFN]-γ) reactivity following intravaginal challenge with *Candida* antigen. Cervicovaginal lavage fluids were collected initially (time 0) and either 16–18 or 36–40 h after introduction of *C. albicans* (Ca) antigen or PBS. Cytokine concentrations are expressed as pg/mg protein lavage fluid. Ranges of concentrations for IL-2 (pg): subjects given PBS and tested at 16–18 h (n = 3), time 0, 226–470; time 16–18 h, 253–1070; subjects given Candida antigen and tested at 16–18 h (n = 5), time 0, 58–115; time 16–18 h, 65–14,588; subjects given PBS and tested at 36–40 h (n = 3), time 0, 117–198; time 36–40 h, 172–367; subjects given Candida antigen and tested at 36–40 h (n = 5), time 0, 28–122 pg; time 36–40 h, 50–210 pg. Ranges of concentrations for IL-12 (pg): subjects given PBS and tested at 16–18 h (n = 3), time 0, 1.6–5.6; time 16–18 h, 7.3–929; subjects given Candida antigen and tested at 16–18 h (n = 5), time 0, 0–238; time 36–40 h, 47–10,706; subjects given PBS and tested at 36–40 h (n = 5), time 0, 0–207; time 16–18 h, 47–10,706; subjects given Candida antigen and tested at 36–40 h (n = 5), time 0, 4–3–16; time 36–40 h, 7.5–115; subjects given Candida antigen and tested at 36–40 h (n = 5), time 0, 0–238; time 36–40 h, 10–149.
creases (IL-4) and decreases (IL-5) in specific cytokine expression. However, none of the systemic cytokine differences could be attributed specifically to Candida antigens. We presume that these systemic changes reflect either a true nonspecific effect or possibly inherent experimental variation, as was the overall interpretation in our previous study [8]. On the basis of the lack of a distinct pattern for the differences in the present study, we again favor the latter as the most appropriate explanation. Furthermore, while comparisons between the vaginal expression of cytokines with those mediated by PBL in response to Candida antigens revealed both similarities and differences, there were no positive correlations that would support an association or link between systemic and vaginal immune expression.

As in previous reports by other laboratories, immunoglobulins measured in vaginal lavage fluid of RVVC patients showed that Candida-specific IgG and IgA concentrations were not different from those in control subjects [20], whereas Candida-specific IgE was increased during symptomatic infection [21–23]. Interestingly, total IgA and IgG were decreased in RVVC patients. It is unclear what this may imply. Perhaps vaginal infection leads to a general reduction in nonspecific immunoglobulins.

Taken together, vaginal-associated immune reactivity in women with RVVC showed no blatant deficiency or imbalance of overall Th-type cytokine expression compared with control subjects, although the decreases in IL-12 may be significant. Moreover, vaginal CMI expression showed little association with systemic Candida-specific CMI. Although we are confident in the data, caution should be taken not to overinterpret the present data, as this clinical de novo analysis contained uncontrollable limitations in experimental design. First, the dynamics and kinetics of the immune responses during infection were difficult to control, because many of the symptomatic RVVC patients attended the clinic at various times from the initiation of infection. Second, none of the differences observed in cytokine or immunoglobulin expression in the CVL fluid from RVVC patients or controls could be positively attributed to C. albicans antigen(s).

To circumvent these problems, we addressed the possibility of stimulating existing Candida-specific vaginal immunity in normal healthy women by intravaginal challenge with Candida antigen. Candida antigen was chosen instead of live organisms because of ethical considerations. All women were tested dur-
ing the follicular stage of the menstrual cycle to avoid influences of reproductive hormones during these preliminary analyses. Of the three clinical protocols tested, those involving the intravaginal introduction of *C. albicans* HKB did not stimulate any detectable increases in vaginal immune reactivity. On the basis of our results using soluble *Candida* antigen (discussed below), we suspect that this was due to the lack of adherence by the killed organism and subsequent inadequate contact of antigen with the mucosa. Certainly, lack of systemic sensitization did not appear to contribute to the lack of activity, as >85% of participants showed positive PBL proliferation in response to *Candida* antigens.

In contrast to the lack of local responsiveness in women given killed *C. albicans*, the intravaginal introduction of soluble *C. albicans* skin test antigen significantly increased all three Th1-type cytokines (IL-2, IL-12, and IFN-γ) in CVL fluid within 16–18 h. None of the Th2-type cytokines were significantly elevated, although amounts of IL-10 did approach significance (*P* = .07) in subjects given *Candida* antigen and tested at 16–18 h. If the increase of cytokines in vaginal secretions reflects the stimulation of a normal protective response in the vagina to *C. albicans*, our results suggest that Th1-type CMI may be an integral component of this response, similar to that described for animal models of mucosal and systemic *C. albicans* infections [13, 14, 33–35].

Interestingly, subjects tested 36–40 h after intravaginal antigen administration showed no local increase in cytokines. Assuming that a certain percentage of all subjects given *Candida* antigen had reactivity at 16–18 h, these data suggest that the reactivity is transient following introduction of antigen. Alternatively, the lack of cytokine expression may have been due to the increased presence of reproductive hormones at 36–40 h as the women approached the preovulatory stage of the menstrual cycle. There is increasing evidence that reproductive hormones negatively influence immune reactivity at both the systemic and cervicovaginal level [24, 37–40].

In contrast to the elevation in cytokines following intravaginal antigen administration, we were unable to detect increases in *Candida*-specific IgA, IgG, or IgE 16–18 or 36–40 h after *Candida* antigen challenge. These results are consistent with the lack of IL-4, which is required for IgA and IgE synthesis [41–43]. Moreover, the lack of increased immunoglobulins may also reflect the fact that B lymphocytes are not considered resident cells of the vaginal mucosa [25]. It is possible that

Figure 5. IgE and histamine following intravaginal challenge with *Candida* antigen. Vaginal lavage fluids were collected initially (time 0) and either 16–18 or 36–40 h after intravaginal introduction of *Candida* antigen or PBS. Ranges for total IgE (U/mg protein): subjects given PBS and tested at 16–18 h (n = 3), time 0, 0–716; time 16–18 h, 0–462; subjects given PBS and tested at 36–40 h (n = 3), time 0, 258–494; time 36–40 h, 384–493; subjects given *Candida* antigen and tested at 16–18 h (n = 5), time 0, 88–1115; time 16–18 h, 113–1238. Ranges of histamine (pg/mg protein): subjects given PBS and tested at 16–18 h (n = 3), time 0, 88–1115; time 16–18 h, 113–1238. Ranges of histamine (pg/mg protein): subjects given PBS and tested at 16–18 h (n = 3), time 0, 88–1115; time 16–18 h, 113–1238. Ranges of histamine (pg/mg protein): subjects given PBS and tested at 16–18 h (n = 3), time 0, 88–1115; time 16–18 h, 113–1238. Ranges of histamine (pg/mg protein): subjects given PBS and tested at 16–18 h (n = 3), time 0, 88–1115; time 16–18 h, 113–1238. Ranges of histamine (pg/mg protein): subjects given PBS and tested at 16–18 h (n = 3), time 0, 88–1115; time 16–18 h, 113–1238. Ranges of histamine (pg/mg protein): subjects given PBS and tested at 16–18 h (n = 3), time 0, 88–1115; time 16–18 h, 113–1238. Ranges of histamine (pg/mg protein): subjects given PBS and tested at 16–18 h (n = 3), time 0, 88–1115; time 16–18 h, 113–1238. Ranges of histamine (pg/mg protein): subjects given PBS and tested at 16–18 h (n = 3), time 0, 88–1115; time 16–18 h, 113–1238. Ranges of histamine (pg/mg protein): subjects given PBS and tested at 16–18 h (n = 3), time 0, 88–1115; time 16–18 h, 113–1238.
longer lavage intervals would allow more time for antigens to migrate to B lymphocyte areas of the lymph nodes and for plasma cells or immunoglobulins to circulate back to the vaginal mucosa.

The increased levels of vaginal histamine 16–18 h after intravaginal challenge with C. albicans antigen is an important observation. First, it provides indirect evidence that fixed Candida-specific IgE was present in the vagina, even though no increases in IgE were detected. Second, it provides additional evidence that the antigen did in fact elicit a response.

Taken together, our data suggest that Th1-type vaginal-associated CMI can be stimulated in normal healthy women 16–18 h after intravaginal challenge with Candida antigen. It should be stressed, however, that the positive results obtained using the soluble intravaginal antigen challenge must be considered preliminary because of small sample size and require confirmation, despite the fact that several protocols were tested that used different antigen preparations and time intervals after intravaginal antigen administration. It remains possible that with increased sample size, increased concentration of antigen, altered intervals between intravaginal challenge and sample collection, and additional exclusion criteria (e.g., exclusion of women receiving hormone replacement therapy or oral contraception, or those whose PBL do not respond to Candida antigen), the vaginal cytokine response will be stronger and be accompanied by elevations in Candida-specific immunoglobulins.

In summary, this is the first reported evidence for the detection and measurement of Th1- and Th2-type cytokines in CVL fluid from normal and vaginally infected women. Despite our lack of identifying a clear deficiency of or imbalance in Th-type immunity in vaginal secretions from RVVC patients and recognizing some obvious limitations in the experimental design, we report a novel approach for studying vaginal-associated immune reactivity by local administration of Candida antigen. Targeting this intravaginal challenge to RVVC patients 22. Witkin SS, Jeremias J, Ledger WJ. Vaginal eosinophils and IgE antibodies might be stronger and be accompanied by elevations in Candida antigen, the vaginal cytokine response will be stronger and be accompanied by elevations in the local mucosa.

References