MAJOR ARTICLE

Frequency of Interleukin-4 (IL-4) −589 Gene Polymorphism and Vaginal Concentrations of IL-4, Nitric Oxide, and Mannose-Binding Lectin in Women with Recurrent Vulvovaginal Candidiasis

Oksana Babula,[1,2] Gunta Lazdāne,[2] Juta Kroiča,[2] Iara M. Linhares,[1] William J. Ledger,[1] and Steven S. Witkin[1]

[1]Division of Immunology and Infectious Diseases, Department of Obstetrics and Gynecology, Weill Medical College of Cornell University, New York, New York; and [2]Department of Obstetrics and Gynecology, Riga Stradin's University, Riga, Latvia

Background. A C→T substitution at position −589 in the interleukin-4 (IL-4) gene is associated with increased production of IL-4. Associations between this polymorphism and recurrent vulvovaginal candidiasis (RVVC), as well as vaginal concentrations of IL-4 and the anticandidal compounds nitric oxide (NO) and mannose binding lectin (MBL), were evaluated.

Methods. Vaginal samples obtained by lavage from 42 women with RVVC during the acute stage of the disease and 43 control samples were assayed by enzyme-linked immunosorbent assay for IL-4 and NO metabolites. The −589 IL-4 gene polymorphism was detected by polymerase chain reaction and endonuclease digestion. Data were analyzed by Fisher's exact test, the nonparametric Mann-Whitney and Kruskal-Wallis tests, and Spearman rank correlation. $P < .05$ was considered significant.

Results. *Candida albicans* was identified in 38 patients with RVVC; 3 others had infection due to *Candida tropicalis*, and 1 had infection due to *Candida krusei*. The IL-4 T,T genotype was detected in 59.5% of patients with RVVC and in 7.0% of control subjects ($P < .0001$). The frequency of IL-4*T was 76.2% in patients with RVVC and 23.3% in control subjects ($P < .0001$). The median concentration of vaginal IL-4 was elevated in patients with RVVC, compared with control subjects ($P < .0001$). Conversely, vaginal concentrations of NO metabolites ($P = .02$) and MBL ($P < .0001$) were reduced in patients with RVVC. There was a positive association between IL-4*T homozygosity and vaginal IL-4 levels ($P < .0001$) and negative associations between this genotype and vaginal NO ($P = .01$) and MBL ($P < .0001$) concentrations.

Conclusions. Reduced vaginal levels of anticandidal factors in IL-4*T homozygotes may increase susceptibility to RVVC.

Candida albicans is a dimorphic yeast that inhabits the vagina as a nonpathogenic commensal microorganism in up to 30% of women of reproductive age [1, 2]. Colonization with *C. albicans* can occur during passage of the newborn infant through the birth canal [3]. Therefore, the vast majority of colonized women have readily detectable anti-*Candida* humoral and cell-mediated immunity [4, 5]. In contrast, laboratory animals used in candidal vaginitis–directed research are not normally colonized with *C. albicans* [6]. Therefore, local and systemic anticandidal immune defense mechanisms might be quite different in animals and in humans [2]. In addition, polymorphonuclear leukocytes are the primary vaginal anticandidal immune defense mechanism in experimental animals, but these cells are typically absent from women with vaginal candidal infections [7]. The mechanisms involved in the conversion of *C. albicans* from a commensal to a vaginal pathogen remain incompletely understood.

There has been a lack of agreement on identification of a specific acquired cell-mediated or humoral immune mechanism responsible for development of a symptomatic candidal vaginal infection in colonized women. Because of the large number of factors and pathways that can contribute to the development of a clinically apparent vaginal yeast infection, the absence

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Reprints or correspondence: Dr. Steven S. Witkin, Dept. of Obstetrics and Gynecology, Weill Medical College of Cornell University, 525 E. 68th St., Box 35, New York, New York 10021 (switkin@med.cornell.edu).

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of consistent findings among different investigators is not too surprising. The conflicting data obtained to date have been recently reviewed [8].

A more recent approach to the problem of why certain infections or other disorders occur repeatedly in some women but not in others has been to evaluate possible genetic differences between patients and control subjects. The association between a polymorphism in the gene coding for complement component C3 and enhanced inflammatory responses to *Candida* infections has been reported [9]. A genetic polymorphism in the gene coding for mannose-binding lectin (MBL) that results in decreased MBL levels recently has been shown to occur with increased frequency in women with recurrent vulvovaginal candidiasis (RVVC) [10]. MBL, a component of the innate immune system, binds to mannan residues on *C. albicans* and promotes complement activation and killing [11]. Nitric oxide (NO) is another major component of the innate immune defense against microorganisms [12]. The anti-*Candida* activity of macrophage-derived NO is strongly inhibited by IL-4 [13]. IL-4 is a cytokine product of the Th2 subset of T lymphocytes. It inhibits production of proinflammatory cytokines by Th1 T cells and blocks macrophage activation [14]. A single nucleotide polymorphism consisting of a C→T transition at position −589 in the promoter region of the IL-4 gene has been identified. Possession of the T variant is associated with increased IL-4 production [15].

We hypothesized that the variant IL-4 −589 T allele (IL4*T) is a gene polymorphism associated with RVVC because of increased concentrations of vaginal IL-4 and decreased NO and MBL concentrations.

METHODS

The study group consisted of 42 women aged 18–35 years, examined at Riga First Hospital in Latvia between 2001 and 2002, who had genital itching and/or abnormal vaginal discharge and positive results of culture for *Candida* and a history of at least 4 clinical episodes of culture-positive vulvovaginal candidiasis in the preceding 12 months. The control group consisted of 43 age-matched healthy women without any vaginal complaints and with no history of a vaginal yeast infection. Details of sample collection, demographic information, and the diagnosis of vaginal *Candida* species, bacterial vaginosis, and other vaginal microorganisms in this study population have been reported previously [10]. Briefly, cervicovaginal samples were obtained by instilling 3 mL of sterile saline into the posterior vagina, mixing the saline with secretions, and withdrawing the solution with a syringe. All vaginal lavage samples were centrifuged to separate supernatant and pellet fractions and immediately frozen at −20°C; afterwards, each fraction was shipped on dry ice to Weill Medical College of Cornell University in New York for analysis.

Vaginal NO production was determined by measuring total vaginal nitrite and nitrate concentrations of undiluted samples with a commercial ELISA (StressXpress). The lower limit of sensitivity was 1.35 μmol/L. IL-4 levels were determined by commercial ELISA (BioSource). The lower limit of sensitivity was 0.9 pg/mL. Vaginal MBL concentrations for both patients and control subjects were previously reported [10].

For the IL-4 genetic polymorphism analysis, sample pellets were thawed, washed, and resuspended in a 1% solution of the nonionic detergent Brij 35 in Tris-HCl buffer, pH 7.5, containing 5 mg/mL proteinase K. Cells were lysed by incubation at 56°C for 60 min, and the proteinase K was then inactivated by increasing the temperature to 95°C for 10 min. The lysed samples were diluted 1:5 in 10 mmol/L Tris-HCl that contained 1.5 mmol/L $MgCl_2$; 50 mmol/L KCl; 0.2 mmol/L each of deoxyadenosine triphosphate, deoxycitidine triphosphate, deoxyguanosine triphosphate, and thymidine triphosphate; 1.25 U of Taq DNA polymerase; and 30 pmol of oligonucleotide primers that amplified the polymorphic region at position −589 in the IL-4 promoter [16]. The final volume was 0.05 mL. Amplification conditions were 35 cycles of 95°C for 30 s, 57°C for 30 s, and 72°C for 30 s. The PCR product was digested by incubation at 65°C for 18 h with *Bsm*FI restriction enzyme (New England BioLabs) in buffer provided by the manufacturer. The wild type allele generated a product that was cut into 192- and 60-bp fragments, whereas the mutant product remained uncut at 252 bp. The products of the PCR reaction were resolved by electrophoresis on a 3% agarose gel stained with ethidium bromide.

Genotype and allele frequencies were determined by direct counting and then dividing by the number of chromosomes to obtain allele frequency and by the number of women to obtain genotype frequency. Differences in genotype and allele frequencies between patients and control subjects were analyzed with use of Fisher's exact test. Hardy-Weinberg equilibrium was determined with use of a χ^2 goodness-of-fit test on the basis of expected frequencies. The expected frequencies were calculated with use of the assumption of Hardy-Weinberg equilibrium. Differences in median vaginal concentrations of IL-4, NO, and MBL between patients and controls were analyzed with the nonparametric Mann-Whitney test. The association between IL-4 genotype and vaginal concentrations of IL-4, NO, and MBL were determined by the Kruskal-Wallis test. Correlations between the concentrations of any 2 vaginal compounds were analyzed by Spearman rank correlation. $P < .05$ was considered statistically significant.

RESULTS

The distribution of the IL-4 genotypes at position −589 differed between patients with RVVC and controls (table 1). Homozygosity for the variant IL4*T was observed in 59.5% of patients

Table 1. Frequency of IL-4 gene polymorphisms in women with recurrent vulvovaginal candidiasis (RVVC).

Variable	No. (%) with polymorphism		*P*	RR (95% CI)
	Control	RVVC		
Genotype				
C,C	26/43 (60.5)	3/42 (7.1)	<.0001	0.149 (0.559–0.812)
C,T	14/43 (32.6)	14/42 (33.3)		
T,T	3/43 (7.0)	25/42 (59.5)	<.0001	2.994 (1.970–4.549)
Allele				
C	66/86 (76.7)	20/84 (23.8)		
T	20/86 (23.3)	64/84 (76.2)	<.0001	3.276 (2.191–4.898)

with RVVC, compared with 7.0% of control subjects ($P <$ .0001). Conversely, homozygosity for IL4*C was present in 60.5% of control subjects and 7.1% of patients with RVVC ($P <$ .0001). The frequency of the IL4*T allele was 76.2% in patients with RVVC and only 23.3% in control subjects ($P <$ .0001). The frequencies of IL-4 genotypes in both patients with RVVC and control subjects were in Hardy-Weinberg equilibrium.

Four patients with RVVC had results positive for *Candida* species other than *C. albicans:* 3 had results positive for *Candida tropicalis,* and 1 had results positive for *Candida krusei.* Two of the women with *C. tropicalis* infection and the woman with *C. krusei* infection were IL4*T homozygotes; the other patient who was infected with *C. tropicalis*- was a IL4*T/IL4*C heterozygote. Demographic and clinical characteristics of the patients with RVVC in relation to their IL-4 genotype are shown in table 2. The only significant difference noted was a higher percentage of smokers among IL4*C homozygotes than among the other 2 groups ($P = .02$). Four of the control subjects also had cultures positive for *C. albicans.* All were IL4*C homozygous.

Vaginal concentrations of IL-4 and NO in both patient and control groups are shown in table 3. Median IL-4 levels were higher ($P <$ .0001) and median NO levels were lower ($P =$ .02) in patients with RVVC than in controls. There were no differences in vaginal IL-4 and NO levels in the RVVC group between those with results positive or negative for *Candida* species other than *C. albicans* or between those with results positive or negative for bacterial vaginosis (data not shown).

The relationship between IL-4 genotype and vaginal concentrations of IL-4 and NO is shown in table 4. Homozygosity for the variant IL4*T allele was associated with elevated vaginal concentrations of IL-4 ($P <$ .0001). Conversely, possession of this genotype was associated with reduced vaginal concentrations of NO ($P = .01$).

There was an inverse correlation between vaginal concentrations of IL-4 and NO (Spearman $\rho = -.257$; $P = .02$).

DISCUSSION

Results of the present study, as well as results of our previous investigation, highlight a genetic influence on susceptibility to RVVC. Possession of variant alleles associated with reduced vaginal concentrations of MBL [10] or increased vaginal IL-4 levels occur at a higher rate in women with RVVC than in a control population. The number of subjects in our study group was insufficient to analyze whether simultaneous carriage of both gene polymorphisms was associated with a further increase in the rate of RVVC. Nevertheless, the data from the 2 investigations strongly support the hypothesis that a woman's genetic capacity to produce IL-4 and MBL influences her likelihood of developing RVVC.

IL-4 blocks macrophage-mediated anti-*Candida* responses,

Table 2. Association of IL-4 genotype with clinical and demographic data in women with recurrent vulvovaginal candidiasis.

Parameter	C,C ($n = 3$)	C,T ($n = 14$)	T,T ($n = 25$)
Age, mean years (range)	23.0 (18–29)	27.8 (19–35)	26.7 (18–35)
Pregnancies, mean no. (range)	2.3 (0–5)	1.8 (0–4)	2.8 (0–8)
Births, mean no. (range)	0.3 (0–4)	1.1 (0–3)	1.2 (0–3)
Married	1 (33.3)	8 (57.1)	11 (44.0)
Smoker	3 (100)[a]	3 (21.4)	6 (24.0)
College education	0	8 (57.1)	13 (52.0)
Gram-positive bacteria	0	2 (14.3)	6 (24.0)
Gram-negative bacteria	0	4 (28.6)	2 (8.0)
Presence of *Urea urealyticum*	1 (33.3)	2 (14.3)	6 (24.0)
Bacterial vaginosis	2 (66.7)	4 (28.6)	4 (16.0)

NOTE. Unless indicated otherwise, data are no. (%) of patients with the specified parameter.

[a] $P = .02$ vs. C,T and T,T.

Table 3. Vaginal concentrations of IL-4 and nitric oxide metabolites in women with recurrent vulvovaginal candidiasis (RVVC).

Compound	Median concentration (range)		
	Control subjects (n = 42)	Women with RVVC (n = 40)	P
IL-4, pg/mL	<0.9 (<0.9–1.5)	2.5 (<0.9–11.2)	<.0001
Nitric oxide, μmol/L	10.2 (<1.3–42.5)	4.0 (<1.3–66.1)	.02

at least in part, by the inhibition of NO production [13]. In the present study, homozygous carriage of IL4*T was associated with both a >2-fold increase in the vaginal concentration of IL-4 and a 3-fold decrease in vaginal NO production. MBL vaginal levels in the study population were reported previously [10], and IL4*T homozygosity was also associated with a >2-fold decrease in the median vaginal MBL concentration (7.3 ng/mL vs. 15.5 ng/mL for IL4*C homozygotes). Similarly, there was a strong inverse relationship between vaginal concentrations of IL-4 and MBL (Spearman $\rho = -.728$; $P < .0001$). MBL is an acute-phase reactant, and it is possible that a macrophage-activation product induces vaginal MBL production. The cells in the female genital tract that produce MBL have yet to be identified. It appears that a woman's IL-4 genotype at position −589 influences the vaginal concentrations of 2 major components of the innate immune response against infection. The data, therefore, also highlight a central role for innate immune mechanisms in protection against development of symptomatic *Candida* vaginitis.

The results of our genetic studies offer new insights into the factors that influence conversion of *Candida* from a commensal microorganism to a vaginal pathogen. The elaboration of a proinflammatory anti-*Candida* cytokine response by healthy women results in the recruitment and activation of macrophages that eliminate or reduce the vaginal *Candida* concentration. The presence of MBL receptors on macrophages allows these cells to bind and ingest *Candida* that have become associated with MBL present in the vagina. The release of NO by activated macrophages results in the production of toxic radicals that kill *Candida*. Activated macrophages release proinflammatory cytokines that stimulate Th1 lymphocytes to further inhibit proliferation of *Candida*. Th1 cells release IFN-γ, which blocks the capacity of *C. albicans* yeast cells to initiate germ tube formation and convert from the yeast to the more invasive fungal form [17]. Macrophage and T cell activation also fosters the prolongation of antimicrobial responses. An enhancement of production of anti-inflammatory cytokines in the vagina, possibly due to a genetic propensity to produce IL-4 and/or other anti-inflammatory mediators or to decreased levels of MBL, induction of a vaginal allergic response accompanied by histamine release [18, 19] and production of prostaglandin E_2 [19, 20] or the concomitant presence of microbial or nonmicrobial Th2 inducers diminishes the ability to limit proliferation of *Candida*. If *Candida* is present as a commensal microorganism, these conditions would allow the replication and germination of the yeast and lead to a symptomatic infection.

Table 4. Association between IL-4 gene polymorphism and vaginal concentrations of IL-4 and nitric oxide (NO) metabolites.

IL-4 genotype	Median concentration (range)	
	IL-4, pg/mL	NO, μmol/L
C,C	0.9 (<0.9–5.7)[a]	12.4 (<1.3–57.3)[b]
C,T	1.2 (<0.9–11.2)	4.3 (<1.3–42.5)
T,T	2.2 (<0.9–9.1)	3.9 (<1.3–66.1)

[a] $P < .0001$.
[b] $P = .01$.

It can be seen that RVVC is the end result of several different pathways. Treatment of a woman's current *Candida* infection will alleviate her symptoms, but this will do little to decrease her susceptibility to developing another symptomatic infection. Because local and systemic azoles are static (not cidal) drugs, these patients are dependent on local host defense mechanisms to eliminate a vaginal candidal infection. Identification of the specific underlying systems in individual women with RVVC that are responsible for a decreased efficiency in killing of *Candida* and initiating specific measures to counteract the observed deficiency will lead to more-effective and longer-lasting treatments.

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