DOI 10.1007/s10517-020-04771-3

IMMUNOLOGY AND MICROBIOLOGY

Differential Expression of Local Immune Response Genes in the Vagina: Implication for the Diagnosis of Vaginal Infections

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 168, No. 11, pp. 588-592, November, 2019
Original article submitted July 24, 2019

Transcription profiles of genes of local immune response were determined in the vagina of women with bacterial vaginosis, aerobic vaginitis, and vulvovaginal candidosis for detection of the most specific immune markers for these vaginal infections. Laboratory diagnosis of the vaginal infections was performed microscopically; the inflammatory reaction in the vagina (leukorrhea) was defined as the presence of >10 white blood cells per field of view. Transcription profiles of *IL1b*, *IL10*, *IL18*, *TNFα*, *TLR4*, *GATA3*, and *CD68* were determined using reverse-transcription quantitative real-time PCR. The strongest predictors of aerobic vaginitis were increased levels of *IL1b* and *IL10* mRNA. Bacterial vaginosis was strongly associated with reduced levels of *IL18* and *GATA3* mRNA. Increased levels of *IL1b* and *TLR4* transcripts showed significant discriminatory power for vulvovaginal candidosis and leukorrhea. The results of this study suggest differential expression of local immune response genes in the vagina of women with different vaginal infections. Detection of specific immune markers in the vagina using reverse-transcriptase PCR could supplement PCR detection of abnormal vaginal microflora for the diagnosis of vaginal infections.

Key Words: *vaginal infections; inflammation markers; transcription profiles; reverse transcriptase; quantitative reverse transcription PCR*

Inflammatory and non-inflammatory vaginal infections, in particular bacterial vaginosis (BV), aerobic vaginitis (AV), and vulvovaginal candidosis (VVC) caused by opportunistic microorganisms are very common in premenopausal women and account for most cases of vaginal discharge [7,11,15]. Along with psychosexual problems, these infections can lead to gynecological and obstetrical complications [1,13].

BV (microbial imbalance in the vaginal microbiota) is characterized by a significant depletion of normal *Lactobacillus*-dominated microbiota and excessive growth of predominantly anaerobic organisms (*e.g*. *Gardnerella vaginalis*, *Prevotella* spp., *Atopobium vaginae*, and *Mobiluncus* spp.), which leads to an increase in vaginal pH and malodorous discharge. Decreased level of lactobacilli and elevated pH are also characteristic of AV, but aerobic microorganisms, like *E. coli*, group B streptococci and *Staphylococcus aureus* predominate in this condition. Clinical presentation of AV includes purulent discharge, some degree of atrophy, and vaginitis. VVC manifested as

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0007-4888/20/1685-0646

curdy-white discharge, vulval and vaginal soreness/itching, and erythema and in ~90% women is caused by excessive growth of *Candida albicans* (remaining cases are caused by other species of yeast-like fungi, *e.g. Candida glabrata*) [7].

Many symptoms and signs of the vaginal infections are unspecific and can accompany other conditions *e.g*. vulval dermatoses of allergic reactions. Microscopy of vaginal discharge is now the main method of laboratory diagnostics of these conditions [13]. In recent years, PCR-based methods for detection of abnormal vaginal microflora characteristic of BV and AV were developed [9,12,14]. Evaluation of mRNA levels of local immune response markers in vaginal samples obtained for PCR characterization of vaginal microflora could supplement microbiological patterns of vaginal infections [2].

Our aim was to determine transcription profiles of local immune response genes in the vagina with the focus on discriminatory ability of the immune markers of different vaginal infections.

MATERIALS AND METHODS

The study participants were women of reproductive age examined in a gynecological clinic in St. Petersburg in March 2017 to November 2018. The main complaints were vulvovaginal symptoms. During examination, the following vulvovaginal signs and symptoms were revealed: abnormal vaginal discharge, vulval soreness/itching and erythema, malodor, dysuria, dyspareunia, purulent or mucopurulent endocervical exudate, cervical petechiae, and abdominal pain. The exclusion criteria were pregnancy, treatment with oral or topical antimicrobials and topical probiotics within 4 weeks prior to enrollment, detection of agents of sexually transmitted infections associated with cervicitis (*Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Trichomonas vaginalis*, *Mycoplasma genitalium*, and HSV), signs of cervicitis, diagnosed or suspected pelvic inflammatory diseases, the use of an intrauterine device or contraceptives delivered directly to the vaginal mucosa. The study was approved by the Ethical Committee of the D.O. Ott Research Institute of Obstetrics, Gynecology, and Reproductology (protocol No. 78/2016). Informed consent was obtained from all individual participants included in the study.

From each woman, two vaginal samples were collected using Dacron swabs. One sample was transferred to a slide, stained after Gram, and examined under a light microscope. The other swab was used for molecular testing: the swab was placed in a tube with 0.5 ml transportation medium (DNA Technology), thoroughly stirred, pressed against the tube wall, and discarded. After transportation within 2-8 h at ambient temperature, the samples were immediately tested or otherwise stored frozen until testing at -20°C for up to 3 months. BV was diagnosed by assessing bacterial morphotypes in Gram-stained vaginal preparations using the Nugent scoring system [6]. Vaginal microflora was classified as normal (a score of 0 to 3), intermediate (4 to 6), or BV (7 to 10). AV was diagnosed using AV scores [6] that combine information about bacterial microflora, leukocyte count, fraction of toxic leukocytes and parabasal cells: 0-2 (no AV), 3-4 (mild AV), 5-6 (moderate AV), or 7-10 (severe AV). The diagnosis of VVC was based on a combination of clinical signs (vaginal discharge, vulval soreness/itching and erythema, dysuria) and microscopic findings (presence of pseudohyphae/mycelia). Leukorrhea was defined as the presence of ≥10 white blood cells per high-power field at ×1000 on microscopic examination.

Transcription profiles of local immune response genes were determined using an mRNA Profiling kit (ImmunoKvanteks; DNA Technology). The kit is based on quantification of *IL1b*, *IL10*, *IL18*, *TNFa*, *TLR4*, *GATA3*, and *CD68* mRNA using reverse-transcription quantitative real-time PCR. The genes were selected from 24 candidate genes of innate immune response based on their level of transcription (mRNA concentration $>10^3$ copies/ml) in vaginal fluid of 310 non-pregnant women of reproductive age [4]. The reference transcript used for normalization was of beta-2-microglobulin mRNA. The analysis was performed in accordance with manufacturer's instructions.

Statistical analysis was performed with the use of MedCalc Statistical Software 18.11 (MedCalc Software bvba; https://www.medcalc.org; 2018). All continuous variables (age, mRNA levels) were first tested for normal distribution using Shapiro—Wilk test, and after being characterized as not normally distributed, were analyzed using Kruskal—Wallis test followed by post-hoc pairwise comparisons using Conover's test. To assess the discriminatory ability of the immune markers for the vaginal infections and leukorrhea, ROC-curves were plotted and classification performance characteristics (areas under curves, Youden's index, and optimum cut-off values) were computed. All tests for significance were two-sided, and the differences were statistically significant at $p<0.05$.

RESULTS

Of 137 examined, 6 women were excluded (two were positive for HSV, one had signs of cervicitis, and three had abdominal pain). Vaginal infections were diagnosed in 37 of 131 enrolled women (28.2%). AV (mild to severe forms), BV, and VVC as isolated infections were detected in 14 (10.7%), 9 (6.9%), and 7 women (5.3%), respectively. Combination of AV and

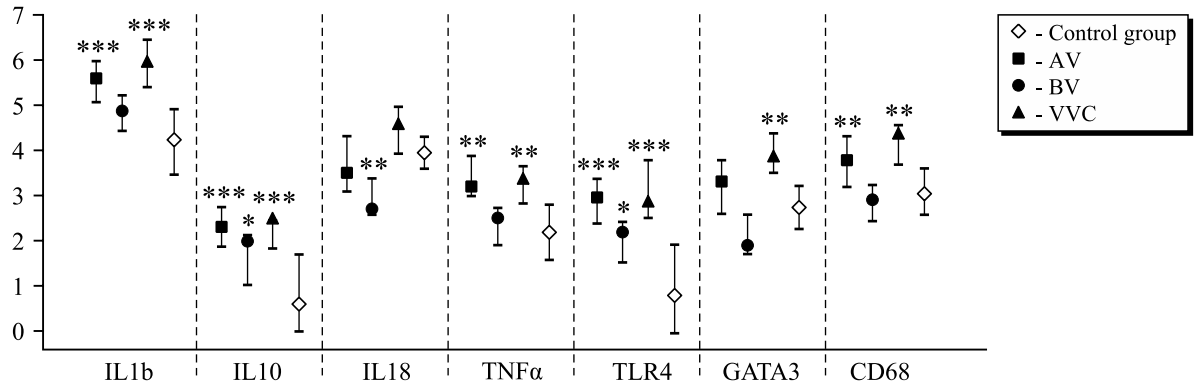

Fig. 1. mRNA levels (log-transformed number of copies/ml) of selected genes of local immune response in the vagina of women with AV, BV, and VVC in comparison with the control group. **p*<0.05, ***p*<0.01, ****p*<0.001 in comparison with the control group.

BV was found in 4 women (3.1%), BV and VVC in one woman (0.8%), AV, BV and VVC in 2 women (1.5%). This, the overall rates of AV, BV, and VVC were 15.3% (*N*=20), 12.2% (*N*=16), and 7.6% (*N*=10), respectively. In 94 women, no vaginal infections were diagnosed, and in 22 of them no vaginal symptoms or signs were recorded. These 22 women were assigned to the control group. There was no difference by the age between the three groups of women (women with vaginal infections, control group, and symptomatic patients, but without vaginal infections): median ages 31, 30, and 31 years, respectively (*p*=0.5).

There were significant differences between the transcription profiles of the selected genes in the groups of patients diagnosed with AV, BV, or VVC alone in comparison with the control group (Fig. 1). The levels of *IL1b* and *IL10* mRNA in samples obtained from women with AV and VVC were significantly higher than in the control group. Regarding BV samples, the levels of expression of these two interleukins were elevated in comparison with control samples, but for *IL1b* the difference did not reach statistical significance. The levels of *IL18* mRNA were significantly lower in women with BV as compared to control women, whereas no difference was observed when AV or VVC samples were compared to the controls. The levels of expression of *TNFα* and *CD68* were significantly higher in women with AV and VVC, and no difference was observed for BV samples. The expression of the *GATA3* gene was significantly increased in women with VVC.

Leukorrhea was found in 56 women, while in 75 women, leukocyte counts did not exceed 10 per high power field. The expression of *IL1b*, *IL10*, *TNFα*, and *TLR4* were significantly higher in women with leukorrhea than in women without it (Fig. 2).

Using ROC analysis, we estimated the capacity of the selected immune markers to discriminate all positive cases of each infection (including cases when this infection was combined with other infections) and leukorrhea among enrolled women. AV was found in 20 women, while 111 women were negative. BV was

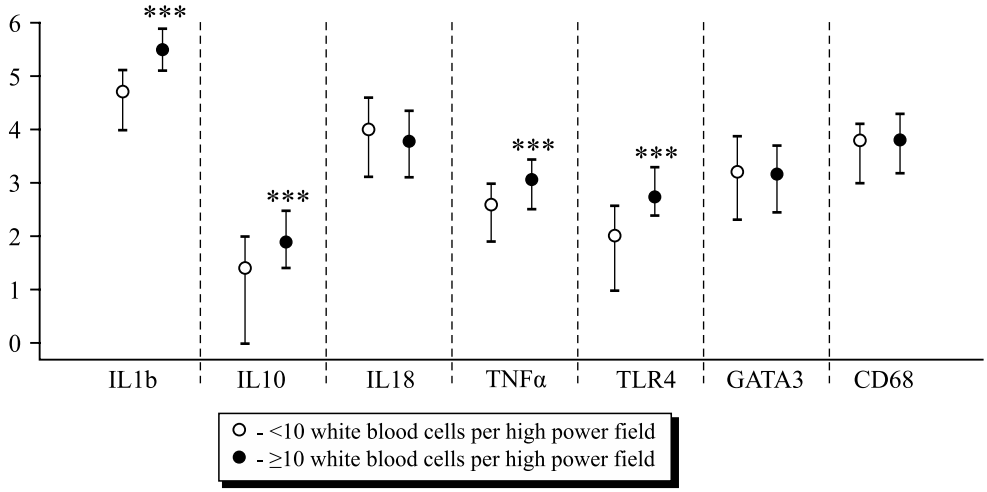

Fig. 2. mRNA levels (log-transformed number of copies/ml) of selected genes of local immune response in the vagina of women with leukorrhea (≥10 white blood cells per high power field) and without leukorrhea (<10 white blood cells per high power field) as compared to women without leukorrhea (<10 white blood cells per high power field); **p*<0.05, ***p*<0.01, ****p*<0.001.

TABLE 1. Discriminatory Power of Selected Markers of Local Immune Response in AV, BV, VVC, and Leukorrhea by the Results of ROC Analysis

Immune markers	Area under curve (Youden's index; optimum cut-off)			
	AV	BV	VVC	Leukorrhea
IL1b	0.749 (0.423; >4.9)	NS	0.788 (0.489; >5.3)	0.779 (0.466; >5)
IL10	0.777 (0.440; >1.6)	NS	NS	0.678 (0.296; >2.2)
IL18	0.679 (0.313; ≤3.7)	0.838 (0.583; ≤2.8)	NS	NS
TNFα	0.689 (0.385; >2.9)	NS	NS	0.695 (0.291; >3.2)
TLR4	0.733 (0.398; >2.7)	NS	0.717 (0.388; >2)	0.754 (0.444; >2.3)
GATA3	NS	0.783 (0.526; ≤2.3)	NS	NS
CD68	NS	NS	NS	NS

Note. NS — area under ROC-curve did not differ significantly from 0.5, which indicated the absence of discrimination power.

found in 16 and was absent in 105 women (10 women with intermediate microflora were not included); VVC was present in 10 and absent in 121 women. Leukorrhea was revealed in 56 and was absent in 75 women. The strongest predictors of AV were increased levels of *IL1b* and *IL10* mRNA (Table 1). BV was strongly associated with decreased levels of *IL18* and *GATA3* mRNA. Increased levels of *IL1b* and *TLR4* showed strong discriminatory power for VVC and leukorrhea.

Changes in the composition of the vaginal microflora, even minor ones, induce local immune response, which modulates the levels of local immune molecules produced by resident immune cells (macrophages, dendritic cells, neutrophils, and natural killer cells) and by vaginal epithelial cells [3,10]. Previous studied aimed at evaluation of the expression of immune response genes (*IL1b*, *IL6*, *IL8*, *IL10*, and *TNFα*) in the lower genital tract of women with normal and disturbed microflora yielded ambiguous results [5,8]. This can be explained by different methods used and variation in composition of the microbial community.

Molecular approaches for defining abnormal vaginal microflora and associated vaginal disorders possess several major advantages over traditional microbiological methods. These include the ability to detect fastidious or uncultivated bacteria, higher objectivity, testing of self-collected samples, possibility of standardization, accurate quantification, and automatization.

Accordingly, our main focus was estimation of the discriminating capacities of the selected immune genes for AV, BV, VVC, as well as for leukorrhea, rather than analysis of the expression profiles of immune molecules and their role in mucosal immunity in different vaginal infections. The selection of the genes was based on their relatively high level of transcription (with mRNA concentration exceeding 10^3 copies/ml) in vaginal fluid of women of reproductive age [4]. We observed significant differences in the expression of these genes in the vagina of women with different vaginal infections, as compared to control women, which was particularly prominent for the *IL1b*, *IL10*, and *TLR4* genes in the infections characterized by inflammation (AV and VVC). At the same time, a dramatic decrease in IL18 expression was characteristic of BV. Leukorrhea was strongly associated with an increase in the expression of *IL1b*, *IL10*, *TNFα*, and *TLR4* genes. In ROC analysis, increased *IL10* levels was the strongest predictors of AV and increased IL1b levels attested to VVC and leukorrhea, whereas reduced levels of *IL18* mRNA had the highest discriminatory ability for BV.

In future studies, we plan to evaluate immune markers together with molecular analysis of relevant microflora in order to reveal the best combinations of immune and microbiological markers, highly sensitive and specific for the vaginal infections. The advantage of using reverse-transcription PCR approach rather than immunological assay is that the analysis for immune markers can be performed using the vaginal samples taken for microflora assessment and on the same PCR based format, after a reverse transcription step.

ImmunoKvanteks kits were kindly provided by the manufacturer, who had no role in development of study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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