Section 3. Medical science

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A STATUS OF CELLULAR IMMUNITY IN BACTERIAL DYSBIOSIS AND BACTERIAL VAGINOSIS

Abstract. Systemic immunodeficiency contributes to the development of bacterial dysbiosis and bacterial vaginosis (BV), therefore, it is relevant to study the systemic response of the immune system, particularly its cellular component, and to search for informative criteria of the pathological process development.

Purpose of the study was to determine the status of cellular immunity by populations of T-lymphocytes in normocenosis, bacterial dysbiosis and BV.

As result in dysbiosis and BV, CD3 and CD4 T-cell immunodeficiency was identified. IRI is a diagnostic factor both for the formation of dysbiosis and for the diagnostics of BV. The model proposed allows us to predict the probability of development of BV with a high level of confidence.

Keywords: bacterial vaginosis, T-cell immunodeficiency.

Bacterial vaginosis (BV) is a common but poorly studied disease of the vagina, which promotes the development of local and systemic immunodeficiency and facilitates the transmission of human immunodeficiency virus (HIV) [1, 219–228], papillomavirus and cervical cancer [2, 9–18; 3, 553–558]. The problem of BV is very important for global health, because vaginal microbiota with high levels of streptococcus or enterobacteria, vaginal candidiasis and trichomoniasis causes an increased risk of pelvic inflammatory disease, pre-term birth and infections for mother and newly born child [4, 859–864]. Over the last decade, an incidence of BV has doubled and ranges from 26% to 40–45% [5, 472; 6]. For example, in the United States BV affects 29% of women, and in sub-Saharan Africa, where HIV is wide spread – 52% of women [7, 505–23].

BV is not usually associated with the redness, swelling, or pain observed in classical inflammation, so it is called “vaginosis”, not “vaginitis” [8, 555–63]. However, BV is related to “subclinical” inflammation of the genitals, which is determined by the hyperre-active response of the immune system [9, 965–76]. According to [10, 156–62], dysbiosis and BV are the main factors contributing to the persistent inflammation of the genital organs and facilitating HIV infection among such women.

It is the lack of local immunity that is an important factor for the occurrence of BV [11, 1399–1405; 12, 103–7]. The reason may be the ability of bacteria forming symbiote in BV to form an active biofilm, which causes suppression of the immune system, chronicity of the process, and resistance to antibi-

otic therapy [13]. In general, dysbiosis and BV are characterized by a decrease of systemic and local inflammatory response [14, 1–5], which, as found in the study [15, 481–7], correlates with an increase of *Gardnerella vaginalis* and *Mycoplasma hominis*.

Therefore, according to the foregoing, it is relevant to study the systemic response of the immune system, in particular of its cellular component, and to search for informative indicators of the pathological process.

Purpose of the study was to determine the status of cellular immunity by populations of T-lymphocytes in normocenosis, bacterial dysbiosis and BV.

Material and methods

This study examined 298 women aged from 16 to 64 who saw gynecologist for a preventive examination or with complaints of genital discomfort of various degrees of manifestation. Subsequent observation excluded patients who had at least one of the definitely pathogenic microorganisms (*Trichomonas vaginalis*, *Neisseria gonorrhoeae*, *Chlamydia trachomatis* and *Herpes Simplex Virus* 1, 2) in the material taken. Presence in the smear of more than 15–20 leukocytes, which indicated of an inflammatory reaction, was also the reason for exclusion from the number of patients.

During the examination, scraping of epithelium from the posterolateral vaginal paries was made using a urogenital probe. Molecular-genetic studies were performed using polymerase chain reaction (PCR) method. DNA was extracted using a kit of reagents “Proba-GS” (“DNK-Technologiia” LLC, RF). Amplification of tubes with the reaction mixture was performed in the amplifier “DTLite” (“DNK-Technologiia” LLC, RF) using the amplification programs recommended by the manufacturer of the reagent kit. Investigation of vaginal biocenosis status was performed using the real-time PCR test system “Femoflor 16”, which allowed to quantify the biota [16, 30] by the following indicators: total bacterial mass (TBM), normobiota (*Lactobacillus spp.*; NB), obligate anaerobes (*Atopobium vaginalis*, *Eubacte-*

rium spp., *Gardnerella vaginalis*, *Prevotella bivia*, *Porphyromonas spp.*, *Lachnobacterium spp.*, *Clostridium spp.*, *Megasphaera spp.*, *Veilonella spp.*, *Dialister spp.*, *Mobilunory spp.*, *Mobiluncus spp.*, *Peptostreptococ spp.*, *Sneathia spp.*, *Leptotrihia spp.*, *Fusobacterium spp.*), Facultative anaerobes (*Enterobacteriaceae spp.*, *Staphylococcus spp.*, *Streptococcus spp.*), mycoplasmas (*Ureaplasma urealyticum + parvum*, *Mycoplasma hominis + genitalium*) and yeast-like fungi (*Candida spp.*).

Criterion for the distribution of patients into groups was an index of conditionally pathogenic microflora (ICPM), which was calculated as the difference between the sum of all conditionally pathogenic microorganisms and the number of lactobacilli in lg GE/sample. In normocenosis ICPM was lower than –3 lg GE/sample (first group, n = 53); in grade I dysbiosis it was from –3 to –1 lg GE/sample (second group, n = 128); and in grade II dysbiosis (BV) it was more than –1 lg GE/sample (third group, n = 117) [17, 54–7; 18, 36–41]. In addition, groups with dysbiosis were subdivided into subgroups by normobiota indicator (NBI), which was calculated as the difference between TBM and lactobacilli (in lg GE/sample). In the 2nd group there are three subgroups: 1st subgroup – with NBI ≤ 0.3 lg GE/sample (n = 23), 2nd subgroup – with NBI from 0.3 to 1.0 lg GE/sample (n = 83), and 3rd subgroup – with NBI > 1 lg GE/sample (n = 22). In the 3rd group there are two subgroups: 1st subgroup – with NBI ≤ 1 lg GE/sample (n = 34), and 2nd subgroup – with NBI > 1 lg GE/sample (n = 83). The maximum degree of dysbiosis was recognized in the 2nd subgroup of the 3rd group, which corresponded to the condition of BV [16].

Subpopulations of T-lymphocytes in the blood were quantified using the method of their visualization in the rosette formation reaction with monoclonal antibodies to T-lymphocytes (CD3), T-lymphocytes helper (CD4), and T-lymphocytes killers/suppressor (CD8) using erythrocyte diagnostic tubes manufactured by “Granum” NPP (Ukraine). Lymphocyte suspension was separated on a density gradient d = 1.077 (“Granum” NPP, Ukraine). The

ratio CD4/CD8 (immunoreactivity index – IRI) was calculated.

For descriptive statistics, arithmetic mean value (M) and standard error of mean (m), median (Me), 1st and 3rd quartiles (Q1; Q3) were used. Variation series were compared using the Student's test (t), single- and multi-factor analysis of variance (criterion F). Paired independent data samples were compared using Mann-Whitney (U) test. Influence of factor variables on the dependent indicators was investigated using linear regression analysis. Regres-

sion coefficients (β), probability of their contrast to the main hypothesis, Wald-statistics value and the maximum probability coefficient for nonlinear models were calculated. Operational characteristics were evaluated using ROC-diagrams. Significance of all the variations was taken at $p < 0.05$. Statistica 10 software package (StatSoft, Inc., USA) was used for statistical processing of the obtained data.

Results and Discussion. Indicators of cellular immunity in patients with normo- and dysbiosis are given in (Table 1).

Table 1. – Indicators of cellular immunity (M $\pm$ m)

Group, subgroup		Leucocytes, G/l	CD3, G/l	CD4, G/l	CD8, G/l	IPI, rel. units
1 st (normocenosis). n=53		6.104 $\pm$ 0.162	1.103 $\pm$ 0.042	0.629 $\pm$ 0.018	0.408 $\pm$ 0.009	1.592 $\pm$ 0.060
2 nd (grade I dysbiosis). n=128	1 st , n=23	6.478 $\pm$ 0.296	1.147 $\pm$ 0.063	0.681 $\pm$ 0.037	0.428 $\pm$ 0.015	1.631 $\pm$ 0.104
	2 nd , n=83	7.142 $\pm$ 0.180	1.165 $\pm$ 0.031	0.690 $\pm$ 0.019	0.414 $\pm$ 0.006	1.695 $\pm$ 0.054
	3 rd , n=22	7.476 $\pm$ 0.337	1.159 $\pm$ 0.058	0.469 $\pm$ 0.020	0.422 $\pm$ 0.015	1.140 $\pm$ 0.061
3-я (grade II dysbiosis). n=117	1 st , n=34	8.658 $\pm$ 0.258	0.595 $\pm$ 0.014	0.442 $\pm$ 0.011	0.414 $\pm$ 0.011	1.094 $\pm$ 0.041
	2 nd , n=83	10.122 $\pm$ 0.183	0.413 $\pm$ 0.007	0.353 $\pm$ 0.007	0.508 $\pm$ 0.009	0.709 $\pm$ 0.017
Statistical procedure of comparison of the results (p)						
p(MW) ¹		0.282	0.527	0.190	0.218	0.709
p(MW) ²		0.001	0.276	0.048	0.758	0.309
p(MW) ³		0.001	0.446	<0.001	0.496	<0.001
p(MW) ⁴		<0.001	<0.001	<0.001	0.811	<0.001
p(MW) ⁵		<0.001	<0.001	<0.001	<0.001	<0.001
F		58.834	126.594	73.392	23.089	72.095
p		<0.001	<0.001	<0.001	<0.001	<0.001

Notes: probability of discrepancies between the corresponding indicators in 1st and 2nd groups using Mann-Whitney test: p(MW)¹ – in the 1st subgroup of the 2nd group, p(MW)² – in the 2nd subgroup of the 2nd group, p(MW)³ – in the 3rd subgroup of the 2nd group, p(MW)⁴ – in the 1st subgroup of the 3rd group, p(MW)⁵ – in the 2nd subgroup of the 3rd group; F – result, and p – probability of the dispersion analysis of variance of differences of the corresponding indicators among the subgroups

Number of leukocytes in the blood under condition of normocenosis was slightly higher than the normal one (4.4–5.5 G/l [19, 960]). As the patients in 1st group did not experience inflamma-

tory processes and/or infectious diseases, we considered these values to be the normal variant.

As dysbiosis progressed, progression of leukocytosis was also observed (Fig. 1). Thus, the content of leukocytes in comparison with the 1st group was in-

creased by 1.2 times in the 2nd group ($p = 0.001$), by 1.4 times in the 1st subgroup of the 3rd group and by 1.7 times in 2nd subgroup of the 3rd group ($p < 0.001$ in both cases). Therefore, under conditions of devel-

opment of BV (2nd subgroup of the 3rd group), leukocytosis acquired the maximum value and exceeded such values in grades I and II dysbiosis (by 1.4 times and 1.2 times respectively; $p < 0.05$ in both cases).

Figure 1. The absolute number of leukocytes in the blood (G/l) depending on the grade of dysbiosis; statistical significance of the differences is shown in Table 1

Figure 2. Content of T-lymphocytes in the blood by fractions (CD3, CD4 and CD8) (G/l; right axis) and IRI value (rel. units; left axis) depending on the grade of dysbiosis; statistical significance of the differences is shown in Table 1

CD3 is a multiprotein membrane complex that is present on the surface of T-lymphocytes and is the major coreceptor of T-cell receptor (TCR) [20, 576]. Due to this, CD3 is a major marker of T-

lymphocytes. As we can see from Table 1, in grade I dysbiosis (2nd group) in comparison with the 1st group (normocenosis) CD3 content in the blood did not actually change, whereas in grade II dysbiosis (3rd

group) it was reduced – by 1.8 times in the 1st subgroup and by 2.7 times in the 2nd subgroup ($p < 0.001$ in both cases).

Such dynamics (Fig. 2) indicated the development of deep grade dysbiosis of CD3-lymphocytopenia. Accentuated decrease in the number of CD3-lymphocytes in the blood was a characteristic of BV, when it was statistically significantly lower than in the case of grade II dysbiosis (by 1.4 times; $p < 0.001$). It indicated the progression of T-cell immunodeficiency with increasing severity of dysbiosis.

CD4 is a monomeric transmembrane glycoprotein that belongs to the immunoglobulin superfamily and is a marker of T-helper cells [20]. It is expressed on thymocytes (80–90%), mature T-lymphocytes (65% of T-helper cells), monocytes, macrophages, Langerhans cells, dendritic cells.

In our study, we observed a significant decrease in the number of CD4-lymphocytes in the blood in severe dysbiosis, namely in the 3rd subgroup of the 2nd group and in the 3rd group, by 1.3–1.8 times ($p < 0.001$ for all these observations). To a maximum extent (by 1.8 times; $p < 0.001$), CD4-lymphocyte content was reduced in BV, when it was significantly lower than in dysbiosis (by 1.3 times; $p < 0.05$). Also, inhibition of T-lymphocytic component in BV was identified [21], which the authors associate with a decrease in predominantly gamma-delta-TcR T-lymphocytes, which recognize non-peptide microbial antigens, similar to NK-cells.

CD8 is transmembrane glycoprotein, which is a coreceptor of T-cell receptors (TCR). Like TCR, CD8 has the ability to bind to a class I major histocompatibility complex molecule [20]. Most of CD8 is represented by peripheral T-lymphocytes as a disulfide-linked alpha-chain homodimer, which is a marker of subpopulation of cytotoxic T-lymphocytes and T-suppressors. On the mature T-cells, either CD8 or CD4 is expressed.

In our study, in normocenosis and dysbiosis content of CD8-lymphocyte in the blood was almost the same (see Table 1 and Figure 2). 2nd subgroup

of the 3rd group was the exception – patients with BV showed a significant increase in content of CD8 lymphocytes in their blood (by 1.2 times compared to those in normocenosis; $p < 0.001$).

Presence of pre-existing trends showing changes in CD3, CD4, CD8 lymphocyte content in the blood, and IRI values in the development of dysbiosis and BV, was confirmed by analysis of variance estimates among the subgroups (see Table 1): F value was from 23.1 to 126.6 ($p < 0.001$ for all the indicators).

Thus, in dysbiosis, there was a gradual, associated with an increase of severity, increase of CD3- and CD4-lymphocytes in the blood, which was expressed to a maximum extent in case of BV. This allowed us to establish the progression of T-cell immunodeficiency in the development of dysbiosis. In contrast, the response of cytotoxic T-lymphocytes and T-suppressors (increase in CD8 content in the blood) was observed only in case of BV, which evidenced the development of compensatory activation of the cellular component of immune system.

The revealed pattern was confirmed and quantified by the dynamics of IRI (see Table 1 and Figure 2). In the 3rd subgroup of the 2nd group and in the 3rd group, IRI was significantly reduced in comparison with normocenosis (by 1.4–2.2 times; $p < 0.001$). Moreover, in case of BV, IRI was reduced to the maximum extent – by 2.2 times in comparison with normocenosis, and in by 1.5–1.6 times in comparison with the indicators in dysbiosis ($p < 0.001$ in all cases).

The analysis of variance (Table 2) confirmed the revealed differences of IRI among the groups of patients with normocenosis (1st group) in comparison with dysbiosis (2nd and 3rd groups). When analyzing the quantitative data, it was found that the value of IRI can be considered a diagnostic marker of development of dysbiosis. IRI is less than $Q_1 = 1.340$ rel. units was the limit below which the dysbiosis could be statistically diagnosed ($F = 20.47$; $p < 0.001$).

Table 2. – Values of IRI in normocenosis and dysbiosis

Indicators	Groups		F	p
	1 st group (normocenosis), n=53	2 nd and 3 rd groups (grade I and II dysbiosis), n=245		
M±m	1.592±0.060	1.222±0.036	20.47	< 0.001
Me (Q1; Q3)	1.543 (1.340; 1.805)	1.081 (0.758; 1.335)		

Notes: $M \pm m$ – mean value and standard error of the mean; Me (Q1; Q3) – median, 1st and 3rd quartiles; F – Fisher's criterion for analysis of variance; p – statistical significance of differences among groups (accepted at $p < 0,05$)

Analysis of the effect of IRI on the development of BV compared to dysbiosis was performed when comparing the data of variance analysis of IRI values in all the subgroups with dysbiosis with the 2nd subgroup of the 3rd group (Table 3).

Table 3. – IRI values in dysbiosis and bacterial vaginosis

Indicators	Groups		F	p
	2 nd group and 1 st subgroup of the 2 nd group (dysbiosis), n=162	2 nd subgroup of the 3 rd group (BV), n=83		
M±m	1.484±0.040	0.709±0.017	186.032	< 0.001
Me (Q1; Q3)	1.401 (1.081; 1.805)	0.700 (0.595; 0.800)		

Notes: $M \pm m$ – mean value and standard error of the mean; Me (Q1; Q3) – median, 1st and 3rd quartiles; F – Fisher's criterion for analysis of variance; p – statistical significance of differences among groups (accepted at $p < 0,05$)

It is established that Q1-Q3 intervals for IRI in groups of patients with dysbiosis and BV do not intersect. In this regard, IRI less than 0.800 rel. units may be considered as a margin, below which there is a probability of BV development ($F = 186.032$; $p < 0.001$).

The fundamental possibility of predicting the development of dysbacteriosis and BV using math-

ematical models of immunity disorders is justified by [22]. According to [23], the need to find optimal and simple prognostic models is an important modern problem, especially in the context of the tendency of BV to become chronic.

Table 4. – Statistical significance of indicators, logistic regression coefficients and their probability for the dependent variable P_{BV}

Indicators	Wald	β -coef.	$\pm SE_{\beta}$	BI±95%	t	p
IPI	46.764	-10.546	1.542	-(13.568-7.523)	-6.826	< 0.001
Independent indicator	46.655	9.305	72.922	6.635-11.976	6.820	< 0.001

Notes: Wald – resulting value of Wald-statistics; β – regression coefficient; $\pm SE_{\beta}$ – regression coefficient error; BI ± 95% – 95% confidence interval; t – Student's coefficient; p – significance of differences compared to the main hypothesis (accepted at $p < 0,05$)

Logistic regression analysis was performed to calculate the probability of BV development. IRI values were used as a factor trait. Presence or absence of BV

was the resulting feature (categorical values “YES” and “NO” were assigned indicator values: “1” and “0” respectively). When developing analysis model,

245 patients with dysbiosis were engaged, 83 patients among them had BV. To evaluate the contribution of IRI values to the probability of BV prediction (P_{BV}), analysis of the resulting Wald-statistics value was conducted. β -coefficients of the regression equation, their standard errors, confidence intervals, and significance of differences compared to the main hypothesis were calculated (Table 4).

Calculated β -coefficients of the regression equation, their standard errors and confidence intervals indicate of the presence of adverse relation of IRI with the probability of BV development ($t = -6,826$; $p < 0.001$ compared with the main hypothesis). Operational characteristics of the developed model, calculated using ROC-analysis, showed the satisfactory quality: $AUC = 0.961 \pm 0.011$ ($CI \pm 95\% 0.940-0.983$; $p < 0.001$). Assessment of the model from the point of view of conformity with the used regression construction method also had satisfactory parameters: $-2\log = 115,426$ ($\chi^2 = 198,281$; $p < 0.001$; $df = 1$). Accuracy of the error-free detection of BV was 91.0%.

Thus, it was determined that IRI is a diagnostic factor both for formation of dysbiosis and for diagnostics of BV. The model proposed meets the quality criteria and allows us to predict the probability of BV development with a high degree of certainty.

Conclusions

1. In dysbiosis on the background of general leukocytosis, there was a gradual, related to the progression of severity of dysbiosis, decrease in the content of blood of CD3- and CD4-lymphocytes, which was expressed to a maximum extent in BV. This allowed us to establish the progression of T-cell immunodeficiency in the development of dysbiosis.

2. The response of cytotoxic T-lymphocytes and T-suppressors (CD8) was observed only in BV. IRI was a diagnostic factor both for the formation of dysbiosis ($IRI < 1.340$ units) and for the diagnostics of BV ($IRI < 0.800$ units).

3. Regression model developed meets the required criteria and allows us to predict the probability of development of BV with an accuracy of 91.0%.

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