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PRODUCTION OF TUMOR NECROSIS FACTOR-A AND ITS SOLUBLE TYPE I RECEPTORS DURING CERVICAL PAPILLOMAVIRUS INFECTION

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Production of tumor necrosis factor-α and its soluble type i receptors during cervical papillomavirus infection. - L. Lazarenko, O. Mykhailenko, L. Ganova, V. Lakatosh, M. Spivak- *Spontaneous promoted production of tumor necrosis factor-α by non-fractioned peripheral blood cells occurs as a result of increased severity degree of disease course during cervical papillomavirus infection in women with cervical intraepithelial neoplasm III and cancer in situ; it is partly due to monocytes activation and higher quantity of $CD8^+$ T-lymphocytes. Simultaneously, the peripheral cell ability to produce the tumor necrosis factor-α is suppressed following additional stimulation by lipopolysaccharide. This cytokine concentration becomes higher in blood and cervical mucus samples, the disease relapses being accompanied by increased level of the tumor necrosis factor-α soluble receptors type I (p55) in blood sera. The synthesis of the tumor necrosis factor-α becomes normalized following combined treatment including the use of a recombinant interferon-α_2 preparation.*

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During these last years, the increase of human papillomavirus (HPV)-induced pre-malignant and malignant diseases of anogenital region was registered in different countries. The HPV was found to be an etiologic factor of cervical cancer (CC) being the second among the most common female tumor nosologies. The HPV structure is now well-known (Poreba, 1998; Burd, 2003). It was also found that a crucial event of the malignant transformation is the interactions of E6 and E7 oncoproteins belonging to high-risk HPV types with intracellular factors being important for cell growth regulation, cell differentiation, and apoptosis (Rorke, 1997; Derchain et al., 1999). At the same time, there are evident data about other factors concerning the human PV infection (PVI) and malignant transformation of HPV-infected cells. In particular, an important role belongs also to the immune defence controlling both viral infection and tumours growth (Stern, 1996; de Jong et al., 2004; Lazarenko et al., 2006). A lot of cytokines including interferon (IFN) types I and II as well as tumor necrosis factor-α (TNFα), IL-2, -4, -5, -10, -18 etc. participate in the development and regulation of immune anti-HPV response (Woodworth et al., 1995).

The TNFα was shown to exert a strong anti-inflammatory effect inhibiting the differentiation and growth of HPV-infected cells, inducing their apoptosis as well as to influence upon the development of the specific anti-HPV response (Viera et al., 1996; Fichorova & Anderson, 1999; Gaiotti et al., 2000; Bachmann et al., 2002). At the same time, some in vitro experiments prove the HVP oncoproteins E6 and E7 regulate (by stimulation or inhibition) the TNFα-induced apoptosis of HPV-transformed cells: these oncoproteins block also cytotoxic action of this compound because of their effect on TNFα soluble receptors type I and II production (Malejczyk et al., 1996, 1997; Lee et al., 2004). It is possible the HPV interaction with the TNFα may influence on the virus persistance, regression or development of HPV-induced disease and HPV-associated pre-malignant and malignant diseases. These opinions were strongly confirmed by some data (Gelder et al., 2003; Govan et al., 2006) suggesting the correlation between patients' sensitivity to PVI and CC associated with HPV with TNFα polymorphism. However, the role of the TNFα and its soluble receptors in HPV-induced immunopathogenesis of pre-malignant and malignant diseases are not yet completely investigated.

Taking all these circumstances into consideration, we had a goal to investigate the TNFα production by peripheral blood cells (PBC) in HPV-infected women as well as in persons with HPV-associated pre-malignant and malignant diseases of different stages; we determined also the TNF and its soluble receptor type I (TNFα-RI, p55) in blood sera and cervical mucus samples.

MATERIALS AND METHODS

We investigated 255 women (their mean age was 26.5) with PVI and PVI-associated cervical pre-malignant and malignant diseases of different stages – benign processes, cervical intraepithelial neoplasm (CIN) I, II, III, and cancer in situ. The HPV DNA detection was made with a polymerase chain reaction (PCR) approach. The PVI diagnosis was carried out using cytomorphology (Meisels & Fortin, 1986; Koss, 1987) and colposcopy (Reid et. al., 1984) data. Our control group included 20 women of the same age healthy from clinical point of view. The group of comparison included 52 patients with urogenital chlamydiosis and 29 women with genital infection carrying the herpes simplex virus type II (HSVII) but without detected HPV.

The TNFα levels in culture media of non-fractionated lypopolysaccharide-(LPS)-stimulated and non-stimulated PBC as well as in blood sera and cervical mucus samples were determined according to their cytotoxic effect in the fibroblast established cell line L-929. The LPS preparation used was from E.coli 026:B6 L-2654 (Sigma, USA). The TNFα levels were calculated taking into consideration their cytotoxicity index (CI, %) according to a formula:

$$CI = \frac{OD(controlAcD) - OD(sample)}{OD(controlAco)} \times 100\%,$$

where OD (control AcD value) is a mean arithmetic of optical density values for control samples AcD; OD of the sample is a mean arithmetic of optical density values for a single sample investigated.

From the difference between IC values in control and LPS-stimulated samples, we evaluated the functional reserve (FR, %) of TNFα-producing cells (Modzolevski et al., 1994). Levels of TNFα-RI (p55) were investigated with an adequate ELISA test-systems produced by the Research Institute of Hematology and Blood Transfusion (Health Ministry of Byelorus Republic) (Petiovka et al., 2003). The phagocytosis activity of peripheral blood mononuclear phagocytes (monocytes) was determined according to the quantity of cells able to absorb test bacteria (S. aureus, strain 209P), i.e. according to their phagocytic number (PhN) as well as taking into consideration the phagocytosis index (PhI), i.e. a mean quantity of test bacteria engorged by a single phagocyte. Oxygen-dependent bactericide activity of peripheral blood monocytes was evaluated by a cytochemical approach using the NTB-test in spontaneous and test bacteria-stimulated (S. aureus, strain 209P) samples (Modzolevski et al., 1994). The monocytes FR (%) was evaluated taking into account the difference between data obtained in the NBT-test for stimulated and non-stimulated cells. Surface antigens of peripheral blood B- and T-lymphocytes were investigated by a direct immunofluorescence approach. Monoclonal antibodies to CD3+, CD4+, CD8+, CD19+ (Becton-Dickinson, USA), CD3+/HLA-DR+ (Immunotech, France) antigens were used. Both T- and B-lymphocytes calculation as well analysis of results obtained were made using a cytofluorimeter FACStar Plus (Becton-Dickinson, USA).

All the data obtained were analyzed by the computer program Epi Info (version 6.0) using a variation statistics approach and Student's criterion. The interactions between different parameters were evaluated using correlation analysis and determining the correlation coefficient (R).

RESULTS

According to the data obtained by Schiffman et al. (1993), the severity of PVI course was classified as light in cases of benign processes (n = 55), moderate in CIN I-II cases (n = 68), and grave ones in cases of CIN III and cancer in situ (n = 45). In patients with benign processes, we identified the HPV-6 and HPV-11 DNA, the HPV types 16, 18, 31, and 33 DNA being found in CIN I-II women; only HPV-16 and HPV-18 DNA sequences were detected in CIN III and cancer in situ women. We found a high frequency of mixed HPV processes with other sexually transmitted pathogens. Chlamydiae were detected in women with benign processes, CIN I-II, CIN III/cancer in situ, the frequencies being 30.1, 36.7, and 28.9, respectively. In some CIN III/cancer in situ cases (8.3 %) HPV associations with the HSVII were detected. In other women, without chlamydiae and HSVII detected, bacterial vaginitis (40.3 %), trychomoniasis (7.4 %), candidiasis (35.2 %) or gonococcus-caused infections (3.7 %) were found. A lot of authors discuss the problem of sexually transmitted accompanying pathogens including chlamydiae and HSVII as risk factors in PVI; so we formed separate patients' groups with PVI-caused single infection and the same infection complicated by chlamydiae and HSVII (mixed infections).

Our results show the interdependence between spontaneous TNFα production by PBC and severity of disease course (see Table I).

In women with benign processes as well as in patients with CIN I-II we showed no changes of TNFα synthesis comparing to control data, the TNFα levels in CIN III/cancer *in situ* patients becoming significantly higher. The FR of TNFα-producing cells determined from the difference between IC values in spontaneous and LPS-stimulated samples did not change in benign process and CIN I-II patients; however, they became almost twice lower in CIN III/cancer *in situ* women. On the other hand, the TNFα concentration in blood sera increased independently on the severity degree of disease course (see Table 1). This cytokine levels became also higher in cervical mucus samples reaching the values 20.9±1.1, 21.8±1.3, and 29.2±1.1 in women with benign processes, CIN I-II, and CIN III/cancer

in situ, respectively, comparing to control levels – 10.2±1.6 ($p = 0.05$). In the majority of PVI (65.0 %), no changes of the TNFα-RI (p 55) levels (2.02±0.10 ng/ml comparing to 2.00±0.10 ng/ml in control samples) were found. However, in relapse cases the p 55 concentration increased up to 3.30±0.09 ng/ml ($p = 0.05$).

Table I. TNFα production by peripheral blood cells and blood sera TNF levels in patients with PVI

Groups of women examined	TNFα production by PBS			TNF level in blood sera IC, %
	spontaneous test, IC, %	LPS-stimulated test, IC,%	FR, %	
Control	12.6±1.0	23.1±2.1	12.2±1.0	26.1±2.3
Benign processes patients (n = 102)	19.2±1.2	29.8±1.2	8.3±1.3	42.3±3.1*
CIN I-II patients (n = 98)	22.1±3.1	31.6±2.1	6.1±0.9	57.3±2.2*
CIN III and cancer *in situ* patients (n = 55)	36.7±3. 4*	39.9±1.5	5.5±1.0*	59.7±3.2*

* – $p = 0.05$ comparing to control values

The results concerning the TNFα production by PBC and its levels in blood sera of mono and mixed infected women carrying chlamydiae and HSVII pathogens are given in the Table II. In patients with benign processes as well as in CIN I-II ones, no changes of spontaneous TNFα synthesis were found. The same data were obtained for mono infected CIN III/cancer *in situ* women; however, during mixed infections the TNFα levels became drastically increased. The FR values of TNFα-producing cells in patients with benign processes and CIN I-II kept control levels becoming significantly lower in CIN III and cancer *in situ* patients both with mono and mixed infections. The TNFα in blood sera increased without any dependence on accompanying chlamydial infection (Table II). In women with urogenital chlamydiosis without HPV detected, the spontaneous TNFα synthesis became also higher (IC = 30.0±1.8; $p = 0.05$). However, following the LPS stimulation no significant increase of the TNFα production was seen (IC = 35.5±3.3) leading to the lower FR value (5.2±0.9; $p = 0.05$). In sera of these patients the TNFα level increased (IC = 49.3±1.3). It was demonstrated the spontaneous TNFα production did not change comparing to mixed infections due to HPV HSVII (IC = 12.8±2.1). Following the LPS stimulation the TNFα synthesis increased insignificantly (IC = 22.3±3.1) accompanied by lower FR value (2.3±0.1; $p = 0.05$). In HSVII-infected patients without PVI detected the spontaneous TNFα synthesis showed no changes (IC = 12.7±3.7). The IC in LPS-stimulated samples increased insignificantly (21.9±9.0), the FR values being without changes comparing to control data (8.1±1.2); however, it was almost thrice lower than in HSVII-infected women without HPV detected. The TNFα levels were increased in blood sera of women with HPV + HSVII association or with the HSVII only (IC values are 40.1±2.1 and 52.5±2.1, respectively). Our results suggest the PVI strengthened with chlamydiae or HSVII influences on the TNFα synthesis in PBC; however, the TNF levels in blood sera became higher both in patients with HPV mono infections and in PVI women infected, in addition, by chlamydiae or HSVII.

Table II. TNFα synthesis by PBC in HPV-infected patients with monoinfection and mixed infection caused by the HPV and chlamydiae

Groups of women examined		TNFα synthesis by PBS			TNF level in blood sera IC, %
		spontaneous test, IC, %	LPS-stimulated test, IC,%	FR, %	
Control		12.6±1.0	23.1±2.1	12.2±1.0	26,1±2,3
Benign processes patients (n = 102)	mono[a]	21.6±4.3	30.7±2.3	9.6±1.5	47.6±1.2*
	mixed[b]	21.9±2.3	34.4±1.2	10.3±1.1	45.1±2.1*
CIN I-II patients (n = 98)	mono	20.4±3.1	27.7±1.4	8.0±1.2	50.1±2.3*
	mixed	24.6±4.3	35.1±1.3	8.5±1.7	57.0±3.5*
CIN III and cancer *in situ* patients (n = 55)	mono	21.5±3.2	26.4±1.9	4.9±0.8*	57.2±3.1*
	mixed	38.1±3.5*	45.1±1.7*	5.3±0.9*	59.3±4.1*

* – $p = 0.05$ comparing to control values; [a] HPV mono infection; [b] mixed infection caused by HPV and chlamydiae

Our results demonstrate also that the increased spontaneous TNFα synthesis at PVI is partially due to monocytes activation. The oxygen-dependent bactericide activity of monocytes in the NBT-test (quantity of NBT-positive cells in spontaneous and stimulated samples, FR) and their PhI did not change comparing to the control; however, we found a tendency to the PhI increase (Table III). In samples from women with benign processes, CIN I-II, and CIN III/ cancer *in situ* no correlation was found between IC and PhI (R = 0.40, 0.13, and 0.17, respectively), quantity of NBT-positive monocytes in the spontaneous test (R = 0.15, 0.19, and 0.14, respectively) and FR (R = 0.15, 0.12, and 0.16, respectively). Simultaneously, a direct correlation was detected in CIN III/ cancer *in situ* women samples between IC and FR (R = 0.59; p = 0.05), such correlation being absent in samples from women with benign processes and CIN I-II (R = 0.11 and 0.15, respectively). On the other hand, the promoted spontaneous TNFα production by PBC is partly due to increased quantity of some T-lymphocyte subpopulations in peripheral blood. The Table IV demonstrates that there is a tendency to lower $CD3^+$ i $CD4^+$ T-lymphocyte quantity and higher $CD8^+$ T-lymphocyte quantities during benign processes, CIN I-II, CIN III/cancer *in situ*, the quantities of leukocytes and lymphocytes being unchanged. The development of severity degree of the disease course was accompanied by decreased $CD3^+$/HLA-DR^+ cell quantity. The $CD19^+$ B-lymphocyte quantity did not change. In cases of benign processes, CIN I-II, CIN III and cancer *in situ* no correlations were found between IC values and quantities of the following cell populations in peripheral blood: $CD3^+$ (R = 0.13, 0.44, and 0.30, respectively), $CD4^+$ (R = 0.12, 0.13, and 0.14, respectively), $CD3^+$/HLA-DR^+ (R = 0.24, 0.20, and 0.04, respectively), $CD19^+$ (R = 0.12, 0.17, and 0.04, respectively). However, the IC value in cases of CIN I-II and CIN III/cancer *in situ* correlate directly with the quantity of $CD8^+$ cells in peripheral blood (R = 0.59 and 0.54, respectively; p = 0.05), such correlation being absent in benign processes (R = 0.13).

Table III. Functional activity of peripheral blood monocytes in patients with papillomavirus infection

Groups of women examined	Quantity of cells per 1 μl				
	NBT-test			PhN, %	PhI, convention-nal units
	spontaneous	stimulated	FR, %		
Control (n = 20)	29.0±2.0 (12.0±1.0)	48.0±2.0 (19.1±1.5)	7.1±1.0	78.0±5.0 (36.0±6.0)	3.4±0.4
Benign processes patients (n = 105)	35.0±7.0 (14.5±1.4)	57±9 (21.9±1.6)	6.4±0.8	102.0±39.0 (47.0±9.1)	3.8±1.3
CIN I-II patients (n = 98)	30.0±9.0 (17.1±1.0)	46.0±9.0 (23.4±1.9)	6.8±0.7	103.0±22.0 (49.1±8.4)	3.7±1.1
CIN III and cancer *in situ* patients (n = 55)	41.0±6.0 (18.0±1.9)	63.0±9.0 (25.1±1.9)	6.5±0.9	138.0±64.0 (49.0±8.2)	3.8±1.1

In parentheses, percents of cells are given.

Table IV. Quantity of peripheral blood lymphocytes in patients with papillomavirus infection

Groups of women examined	Cell quantity per 1 μL						
	leukocytes	lymphocytes	$CD3^+$	$CD4^+$	$CD8^+$	$CD3^+$/HLA-DR^+	$CD19^+$
Control (n = 20)	5520.0±200.0 (100)	1992.0±121.0 (35.6±3.9)	1400.0±99.0 (70.0±2.7)	806.0±99.0 (42.1±2.9)	462.0±90.0 (25.2±2.8)	120.0±11.0 (6.3±0.9)	177.0±10.0 (8.9±1.0)
Benign processes patients (n = 55)	5080.0±60.0 (100)	1900.0±144.0 (36.7±6.2)	1336.0±112.0 (66.8±6.2)	768.0±91.0 (40.5±5.5)	564.0±20.0 (29.2±3.8)	121.0±11.0 (8.8±1.0)	177.0±20.0 (8.4±2.0)
CIN I-II patients (n = 68)	5200.0±98.0 (100)	1860.0±119.0 (35.1±4.8)	1288.0±124.0 (67.9±6.1)	718.0±90.0 (38.6±3.3)	571.0±34.0 (30.0±4.6)	89.0±18.0 (4,7±0,1)	179.0±15.0 (8.1±1.0)
CIN III and cancer *in situ* patients (n = 45)	5201.0±73.0 (100)	1908.0±100.0 (35.9±5.7)	1188.0±116.0 (68.6±5.9)	629.0±68.0 (30.5±3.4)	502.0±96.0 (29.2±3.9)	60.0±9.0 *• (3.0±0.9 *•)	175.0±14.0 (8.9±2.0)

In parentheses, percents of cells are given.

● – $p < 0,05$ comparing to control values;

● – $p < 0,05$ comparing to values in samples taken from women with benign processes.

The TNFα synthesis in PBC and its levels in peripheral blood sera became normalized following the treatment of accompanied infections and inflammation processes in PVI patients (29 women) using a combined approach including a local cytostatic therapy (5-ftoruracil), cryodestruction and with system interferonotherapy (recombinant IFN-α_2) were carried out (Lazarenko, 2006). In three month after such combined therapy the IC values became lower in spontaneous (13.0±2.0 comparing to 36.9±1.5 before treatment; $p = 0.05$) and LPS-stimulated (20.0±3.0 comparing to 36.7±3.4 before treatment; $p = 0.05$) samples, the FR of TNF-producing cells became higher (10.0±1.0 comparing to 6.1±0.9 before treatment; $p = 0.05$). The levels of blood sera TNF decreased (IC = 22.1±2.1comparing to 58.6±3.1 before treatment; $p = 0.05$).

DISCUSSION

It is evident the PVI and associated pre-malignant and malignant cervical diseases change the TNFα synthesis by PBC, the TNF becoming higher in blood sera and cervical mucus samples. In women with grave degree of disease course – CIN III and cancer *in situ* patients infected by high-risk HPV types and by chlamydiae we detected the increased TNFα synthesis by PBC; this fact might have reflect the inflammatory anti-chlamydial reaction of the organism. Such assumption was confirmed by increased spontaneous TNFα production in patients with urogenital chlamydial infection without DNA of HPV detected. The TNFα hyperproduction by PBC at PVI is partly due to monocytes activation and increased $CD8^+$ T-lymphocyte level. However, in high-grade CIN patients Lee et al. (2004) show the decreased quantities of activated $CD4^+$ T-lymphocytes producing TNFα and other Th1-type cytokines (IFN-γ and IL-2). We have also showed earlier (Lazarenko et al., 2002) the IFN-γ production to become suppressed in CIN III and cancer *in situ* patients accompanied by decreased $CD4^+$ T-lymphocyte quantity in peripheral blood. In such a way, the increased TNFα synthesis by non-fractioned PBC taken from CIN III and cancer *in situ* patients occurs simultaneously with suppressed γ-IFN production. It is possible there is disturbance of normal coordination for different Th1-type cytokines production which may impair the development of anti-HPV immune response. This assumption is confirmed be a fact of normalized TNFα and IFN-γ production by PBC: it reaches 4.70±0.09 $\log_2$ U/ml comparing to 2.60±0.10 $\log_2$ U/ml before the treatment and 4.80±0.02 $\log_2$ U/ml in control samples (unpublished data). We have also demonstrated (Lazarenko, 2006) the normalization of other parameters of immune reactivity accompanied by increased therapeutic effect for patients with NU PVI supported by decreased relapse frequency.

On the other hand, our results demonstrate the suppressed PBC ability to produce the TNFα following LPS stimulation in samples taken from PVI patients. The FR values of TNFα-producing cells became lower in CIN III and cancer *in situ* patients both with mono and mixed infections due to HPV and chlamydiae. The same is also true for women infected by HPV and HSVII comparing to control values as well as to patients carrying only the HSVII and to women with urogenital chlamydial infection. The data obtained here are agreed with other results (Lazarenko et al., 2006) demonstrating that chlamydiae and HSVII may be a risk factor of PVI and PVI-associated pre-malignant and malignant cervical diseases. It is thought the PBC producing high cytokine quantities may be activated by their additional stimulation only to certain levels. There is a supposition about the existence of genetically determined limits for leukocyte ability to produce cytokines. In particular, such data are published concerning the PBC of healthy donors (Shcheglovitova et al., 2001). It cannot be, however, excluded the decreased PBC ability to synthesize the TNFα following additional stimulation found in PVI patients may weaken the anti-viral defense of the organism, activate the infection and lead to more severe course of disease. The cause is that the TNFα exerts a direct cytotoxic effect on HPV-infected cells, regulates their growth and proliferation, and induces their apoptosis (Woodworth et al., 1995; Viera et al., 1996, Gaiotti et al., 2000). This cytokine activates also immunocytes of cervical mucus promoting the synthesis of immunoregulating cytokines as well as the expression of HLA class II molecules on epithelium cells (Fichorova & Anderson, 1999).

The TNFα participation in immunopathogenesis processes occurring in PVI women is confirmed also by increased levels of this cytokine in blood sera independently on the severity degree of disease course as well as on mixed infections by HPV and other sexually transmitted pathogens. However, in cases of severe relapsing disease course we have found increased blood sera levels of the TNFα-RI (p55). It is clear the TNFα-RI *in vitro* defend HPV-transformed cells from cytotoxic/cytostatic TNFα effect, the higher TNFα-RI levels in blood sera favouring the development of HPV-associated anogenital loci (Malejczyk et al., 1997; Lee et al., 2004). That is why the increase of TNFα-RI concentrations in blood sera must be interpreted as an unfavourable prognostic marker of PVI course, this marker being independent on other ones determined by methods of immunology and virology.

However, the TNFα role in the PVI process is not unidirectional. For example, some *in vitro*

experiments show the TNFα promotes the expression of HPV-16 oncoproteins, E6 and E7, in keratocytes and stimulate their proliferation (Gaiotti et al., 2000). Woodworth et al. (1995) have detected pro-inflammation cytokines, TNFα and IL-1α, stimulate the proliferation of epithelial cells immortalized by HPV-16 or HPV-18. The TNFα is known to increase the expression of some antigens on cervical mucus; among these antigens are intercellular adhesion molecules – ICAM-1 (Fichorova & Anderson, 1999; D'Anna et al., 2001), VCAM-1, and E-selectin (D'Anna et al., 2001); they promote the adhesive ability of endothelial cells necessary for tumor progression. That is why the increased TNFα concentration in cervical mucus of PVI patients found in our investigations may be an unfavourable marker of the disease course. Azar et al. (2004) show the TNFα concentrations in cervical secrets to be higher in high-grade CIN patients comparing to low-grade CIN ones. The CIN is accompanied by promoted TNFα mRNA expression in HPV-infected NU tissue of high-grade CIN patients comparing to low-grade CIN and control women (Pardo-Govea et al., 2005). We found the increased TNFα concentrations in cervical mucus independently on severity degree of the disease course – in patients with benign processes, CIN I-II, CIN III and cancer *in situ*. However, the IFN level in cervical mucus is lower in CIN III and cancer *in situ* patients comparing to women with benign processes (1.10±0.09 and 2.90±0.01 $\log_2$ U/ml, respectively; p = 0.05; unpublished data).

In such a way, investigations of TNFα production by PBC as well as of TNFα and TNFα-RI levels in blood sera and cervical mucus are important for prognostics and permit to evaluate systemic and local anti-HPV response. However, the interpretation of these results must be carried out taking into consideration the severity degree of disease course, the presence of other PVI-accompanying sexually transmitting pathogens, and changes of organism's cytokine profile and other parameters of immune reactivity.

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