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The Comparison of Immune Indicators and Evaluation of Their Information Loads for the Diagnosis of Different Forms of Lyme Disease

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Abstract: The results of studies of immune and cytokine status in patients with erythemal (n=113) and non-erythemal (n=242) forms of acute Lyme borreliosis were presented. Determinations of correlations were carried out and the proportions of indicators associated with the clinical course of the disease were subjected to quantitative assessment. The immunological criteria for differential diagnosis of erythemal and non-erythemal forms of disease were identified. At the height of the disease, intensive production of IL-4 in the erythemal form and a high concentration of IL-8 in the non-erythemal form of the disease were established. In the convalescence period, increased production of IL-1β and TNF-α in the erythemal form of disease and increased synthesis of IL-8 in the non-erythemal form of Lyme borreliosis were observed. Also differences in expression levels of CD3 molecules, ratios of Th1/Th2 immune response options and functional activity of the phagocytic immune system were revealed. The erythemal form of the disease was characterised by high values of CD3+ cells and average levels of IL-8 production. The non-erythemal form was characterised by the lowest level of IL-8 production and intermediate levels of expression of CD3 and CD4 molecules.

Key words: Lyme borreliosis · Immune status · Cytokines · ANOVA · Factor influence

INTRODUCTION

Lyme borreliosis is a group of transmissible infectious diseases. Their aetiology is similar to that of zoonotic borreliosis and is characterised by clinical phases, polymorphic clinical symptoms and a tendency towards a chronic course of infection [1]. The immune system plays a key role in the pathogenesis and outcomes of Lyme borreliosis. It has been proven that persistence and dissemination of Borrelia has suppressive effects on the immune system. The severity of the defects of the immune system depends on the initial immunoresistance and is directly proportional to the duration and severity of the infection process [2, 3]. However, some results of studies on the types of immune response in different clinical forms of borreliosis, determining the mechanisms of immune-mediated reactions, are contradictory [4, 5]. The research of Simakova et al. (2004) demonstrated the imbalance of pro- and anti-inflammatory cytokines with a predominance of the Th2-type of immune response in patients with acute Lyme borreliosis [6]. The experimental study of the serum of mice infected with *B. burgdorferi* showed Th2-type and mixed Th1/Th2 types of immune response [7]. There is compelling evidence regarding the prevalence of Th1-type immune responses to Borrelia infection: effector Th1-lymphocytes have a direct stimulatory effect on phagocytosis and cytotoxic lymphocytes [4, 8]; also, in patients with Borrelia meningoencephalitis, INF-γ was detected in the cerebrospinal fluid, which also corresponds to Th1-type of immune response [9].

Detailed studies of the functional activity of immune cells, systemic inflammation and prognosis are impossible without determining the levels of cytokines [3, 9, 10, 11]. The erythemal form of Lyme borreliosis is characterised by significantly increased levels of cytokine synthesis-IL-4, IL-10, IL-12p40 and IL-13 - against the background of normal values of IL-12p70, INF-γ and a persistent deficiency of IL-2 throughout the entire disease course. The non-erythemal form of borreliosis is characterised by the increased production of proinflammatory cytokines TNF-α, IL-1α, IL-8 and IL-12p70 in the early stages of

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borreliosis and increased INF-γ production later. However, the production of the anti-inflammatory cytokines IL-4, IL-10 and IL-13 is also increased [6]. Probably, these differences were due to the specific regulation of infectious inflammation, which depends on the immunoreactivity of patients and determines the clinical forms of borreliosis.

Objective: To compare the indices of the immune system and to evaluate their information loads for the differential diagnosis of different forms of Lyme borreliosis.

MATERIALS AND METHODS

We examined 355 patients with acute Lyme borreliosis, who were treated at the infectious department of N.S. Karpovich Krasnoyarsk City Clinical Emergency Hospital during the period 2005-2010. Diagnoses were confirmed on the pathognomonic clinical epidemiological data and the identification of specific IgM and IgG antibodies was also confirmed using ELISA.

The patients were divided into two groups in accordance with the clinical classification. The first (I) group consisted of 113 (31.8%) patients with the erythemal form of Lyme borreliosis, in whom 54 (47.8%) were male and 59 (52.2%) were female; the mean age was 47.9±1.0 years. The second (II) group was represented by 242 (68.2%) patients with the non-erythemal form of Lyme borreliosis and included 122 (50.4%) men and 120 (49.6%) women; the mean age of the patients was 44.8±0.9 years. The control group (CG) consisted of 35 healthy volunteers from blood donors, examined in the Krasnoyarsk Regional Blood Centre. The studied (I, II) groups and the control group were comparable in age ($F=2.7$, $p>0.05$) and gender ($\chi^2=0.2$, $p>0.1$). The patients were examined during the height of borreliosis at the time of admission (d_0) and during the convalescence period to the beginning of the fourth week of disease (d_{21-25}).

We used monoclonal antibodies (anagoges produced by "Becton Dickinson" adapted for use with fluorescence microscopy) to determine the levels of expression of the following lymphocytic molecules: CD3, CD4 and CD8. The absolute numbers of lymphocytes (ALC, μl^{-1}), specified cells (abs, μl^{-1}) and CD4+/CD8+ cells ratios were calculated.

We evaluated the phagocytic index with latex particles (PHI, %) and calculated the number of phagocytised neutrophils (PHN, μl^{-1}). The total concentrations of IgA, IgM and IgG antibodies in the sera were measured by enzyme immunoassay.

The levels of cytokines (TNF-α, IL-1β, IL-4, IL-8) in serum were determined by ELISA. The following reference ranges were considered: for IL-1β<5 pg/ml, for IL-4<6 pg/ml, for IL-8<62 pg/ml and for TNF-α<8.21 pg/ml.

Calculations were carried out using a statistical programs package ("Statistica for Windows 6.0"). The main statistical parameters taken into account were arithmetic mean values (M) and standard errors (m). The difference between rates within the groups was tested using the Mann-Whitney U-test. To assess the relationships between patient indicators, we used the Spearman coefficient of rank correlation (rS). The comparison of proportions was performed using chi-square (χ^2) calculations. The critical significance level (p) for the verification of statistical hypotheses was assumed to be 0.05. In the case of $p<1.0*10^{-29}$, which it was not possible to measure in the statistical software chosen, we used p=0.00.

The applied methods of ANOVA allows the quantitative evaluation of the informativeness of indicators for the subsequent selection of the most important predictors, which is useful for the differential diagnosis and the comparative evaluation of the borreliosis.

The factor's influences were determined by the formula:

$$\eta^2 = \frac{D_f}{D_f + D_r},$$

The following designations were used: η^2 - the power of factor's influence, D_f - factorial dispersion, D_r- random dispersion. [3].

RESULTS AND DISCUSSION

Dynamic changes in cytokine production were revealed, indicating differences in the pathogenic mechanisms of various clinical forms of Lyme borreliosis. In the erythematous form of Lyme borreliosis (I), during the height of the disease, significantly higher levels of investigated cytokines were noted compared with the control group (CG) and these persisted throughout convalescence; the exception was IL-8, which, in the second study, had levels that were comparable to that of the control ($p>0.05$). During the disease, reducing concentrations of IL-1β, IL-8 ($p<0.001$) and TNF-α ($p<0.05$) were registered, relative to baseline values in the group (Tab. 1).

Table 1: Cytokine levels in patients with erythemal and non-erythemal forms of acute Lyme borreliosis (M±m)

Indicators	CG	Grps	D_0	D_{21-25}	P	P_{I-II} D_0	P_{I-II} D_{21-25}
IL-1β (pg/ml)	83.8±7.9	I	651.2±26.7**	504.5±21.3**	<0.05	>0.1	<0.001
		II	708.2±22.0**	385.6±15.6**	<0.001		
IL-4(pg/ml)	47.6±4.0	I	72.1±4.0**	67.7±2.5**	>0.1	<0.05	>0.1
		II	78.4±2.1**	73.4±2.5**	<0.05		
IL-8(pg/ml)	72.9±7.2	I	98.6±5.4*	68.2±3.9	<0.001	<0.001	<0.001
		II	64.0±1.8	94.4±2.5*	<0.001		
TNF-α (pg/ml)	6.8±0.6	I	45.0±1.7**	36.8±1.8**	<0.05	>0.1	<0.001
		II	46.0±1.3**	28.0±1.0**	<0.001		

Note. The significance of differences with the CG: * - p<0.05; ** - p<0.001.

Table 2: The correlations between cytokines in patients with acute Lyme borreliosis

Groups	Indicators	IL-1β	IL-4	IL-8	TNF-α
I	IL-1β	-	0.187* / -0.122	0.022 / 0.116	-0.064 / -0.008
	IL-4	0.187* / -0.122	-	-0.071 / 0.049	0.053 / -0.192*
	IL-8	0.022 / 0.116	-0.071 / 0.049	-	-0.152 / -0.099
	TNF-α	-0.064 / -0.008	0.053 / -0.192*	-0.152 / -0.099	-
II	IL-1β	-	0.198* / 0.039	0.084 / 0.093	0.168* / 0.009
	IL-4	0.198* / 0.039	-	0.125 / 0.313**	0.198* / 0.033
	IL-8	0.084 / 0.093	0.125 / 0.313**	-	0.067 / 0.114
	TNF-α	0.168* / 0.009	0.198* / 0.033	0.067 / 0.114	-

Notes:

1.Table shows the values of rS at the first (d_0) and second (d_{21-25}) examination.

2.The significance of differences with the CG: * - p<0.05; ** - p<0.001.

In the non-erythemal form of Lyme borreliosis (II), in the height of the disease and during convalescence, there was an increase in the synthesis of IL-1β, IL-4 and TNF-α relative to the values of the CG (p<0.001). There was also a significant decrease in the mean values of IL-1β (p<0.001), IL-4 (p<0.05) and TNF-α (p<0.001) relative to their baseline in the group. The level of IL-8 production at the height of disease was comparable with the values in the CG (p>0.05) and increased in the period of convalescence in comparison with the level in the CG (94.4±2.5 pg/ml vs. 72.9±7.2 pg/ml, p<0.05), compared with baseline values (94.4±2.5 pg/ml vs. 64.0±1.8 pg/ml, p<0.001; Tab. 1).

The cytokine status was studied depending on the presence of erythema migrans in the clinical course of the disease (Tab. 1). At the height of the disease, the levels of IL-1β and TNF-α were similar in the patient groups I and II (p>0.1). The IL-4 concentration in the serum of patients with the erythemal form of Lyme borreliosis was lower than in patients with the non-erythemal form (72.1±4.0 pg/ml vs. 78.4±2.1 pg/ml, p<0.05). In contrast, the production of IL-8 in the erythemal form of Lyme borreliosis was higher than in the non-erythemal form (98.6±5.4 pg/ml vs. 64.0±1.8 pg/ml, p<0.001).

During convalescence, the levels of IL-1β and TNF-α were higher in the erythemal form of the disease when compared with the indicators of the non-erythemal form of Lyme borreliosis (p<0.001). The level of IL-8 in group II was higher than in group I patients (94.4±2.5 pg/ml vs. 68.2±3.9 pg/ml, p<0.001). The main differences with the non-erythemal form of Lyme borreliosis, in contrast to the erythemal form, were elevated cytokine IL-4 production at the height of disease and the reduced production of IL-1β and TNF-α during convalescence. In the erythemal form of Lyme borreliosis, there were comparatively higher levels of IL-8 at the height of disease. In contrast, in the non-erythemal form of the disease, a high level of IL-8 was observed in the convalescence period (Tab. 1).

The main effects of IL-4 in patients with different forms of Lyme borreliosis should be considered the activation of production IL-1β, as well as the activation of the production of IL-8 and TNF-α in the non-erythemal form of the disease. The increased production of IL-1β promotes activation of TNF-α production in the non-erythemal form of Lyme borreliosis. The analysis of cytokine production in group I patients showed positive correlations between the levels of IL-1β and IL-4 (p<0.05) at the height of disease and a negative correlation between the levels of IL-4 and TNF-α (p<0.05) during convalescence in patients with the erythemal form of Lyme borreliosis (Tab. 2).

Table 3: The informativeness of immune indicators for the differential diagnosis of erythemal and non-erythemal forms of acute Lyme borreliosis

Indicators	I (n=113)	II (n=242)	Đ I-II	F	p	η^2
ALC, μl^{-1}	1368.9±65.2	1950.6±73.7	<0.001	24.8	$9.8*10^{-7}$	0.12
CD3+, %	55.4±2.3	36.1±0.7	<0.001	108.2	0.00	0.39
PHI, %	53.2±1.6	46.3±1.1	<0.001	12.1	$5.6*10^{-4}$	0.06
PHN, μl^{-1}	2454.2±136.4	1904.3±85.0	<0.001	12.5	$4.6*10^{-4}$	0.06
IL-8, pg/ml	98.6±5.4	64.0±1.8	<0.001	59.2	$1.4*10^{-13}$	0.26

Notes:

1.The table shows the valid values ($p<0.05$).

2.η^2 - the power of influence of characteristic on the values of the indicators (ranging from 0 to 1).

3.In the case of $p<1.0*10^{-29}$ we used p=0.00.

In group II (non-erythemal form of Lyme borreliosis), positive correlations were found between the levels of IL-1β and IL-4 ($p<0.05$), IL-1β and TNF-α ($p<0.05$) and IL-4 and TNF-α ($p<0.05$) during the height of the disease; also, a positive correlation was reported between the production of IL-4 and IL-8 ($p<0.001$) in the convalescence period.

Correlation analysis results show that IL-4 is a key regulator of immune responses in patients with Lyme borreliosis. IL-4 stimulates the proliferation of CD4+ and CD8+ T-lymphocytes and NK-cells, activates the Th2 immune response and triggers the synthesis of immunoglobulins through B-lymphocyte activation [4, 11].

At the height of the erythematous form of disease, positive correlations between IL-1β and IL-4 demonstrated a high activity of macrophages and the early phases of cytokine production during the course of infection [6]. However, the absence of correlations between the values of IL-4 and TNF-α in the erythemal form of Lyme borreliosis at the height of disease and a negative relationship during convalescence indicates a mature immune response, phagocytosis efficiency and switched immune responses to immunoglobulin synthesis. In contrast, in the erythemal form of Lyme borreliosis, the activation of TNF-α production at the same time as the key cytokines - IL-1β and IL-4 - may be an indication of the collapse of phagocytic immune systems at the height of the disease.

It is known that failure of the phagocytic immune system leads to an overload of bacterial antigens, which bind to CD16 (FcγRIII) molecules on the membranes of neutrophils and thereby further inhibit phagocytosis. This increases the binding of antigens with the fourth type of toll-like receptors (TLR4) and activates TNF-α production [3, 10, 11]. It is obvious that, in such clinical situations, the level of TNF-α can be considered a marker of the failure of phagocytosis.

The synthesis of IL-8 is influenced most significantly by the cytokines IL-1β and TNF-α [11]. It is remarkable that the synthesis of IL-8 at the height the disease does not correlate with the synthesis of other cytokines. Activation of attraction mechanisms begins early in the inflammatory process, accompanied by disturbances of microcirculation and clinically manifests as the development of erythema and oedema. Therefore, we can assume that, in patients with the erythemal form of Lyme borreliosis, the processes of attraction has already regressed and regulated at the time of first examination through other mechanisms. A positive correlation between IL-4 and IL-8 production in patients with the non-erythemal form of Lyme borreliosis during convalescence is important. This may be indicative of macrophage activation that occurs as a compensatory response, while reducing the antigenic load and reducing the severity of defects in the phagocytic immune system that are caused by antibiotic therapy. The increased synthesis of IL-8 by macrophages contributes to the emergence of a "second wave" of attraction of neutrophils to the sites of inflammation and the stimulation of immunoglobulin synthesis, involving mechanisms associated with IL-4 and Th2 cells. However, the effectiveness of such reaction mechanisms is uncertain, since the increase in IL-8 production is accompanied by a decrease in the level of IL-4.

Differences in the pathogenesis of Lyme borreliosis between groups I and II are represented by the most informative indicators - CD3+ ($\eta^2=0.39$), IL-8 ($\eta^2=0.26$) and ALC ($\eta^2=0.12$); less informative indicators were PHI ($\eta^2=0.06$) and PHN ($\eta^2=0.06$) (Tab. 3).

The smallest number of indicators which can differentiate these two forms of the disease confirms the unity of the aetiological factors and the substantial similarity of the pathogenic characteristics. Obviously, factors of cellular immunity in conjunction with the condition of chemotaxis (in our study was evaluated by

production of IL-8) and the phagocytic immune system are crucial to the development of various forms of mono-infection in Lyme borreliosis.

Differences between the pathogenetic mechanisms of the immune response in patients with the non-erythemal (II) and the erythemal form (I) of acute Lyme borreliosis are caused by the characteristics of cellular linkage of the immune system: high values of ALC ($p<0.001$) and the low expression of CD3 molecules ($p<0.001$; Tab. 1).

Thus, the erythemal form of acute Lyme borreliosis is characterised by a high value of VCD3 + and an average level of IL-8 production.

The non-erythemal form of acute Lyme borreliosis characterised by the lowest level of IL-8 production and intermediate levels of the expression of molecules CD3 and CD4, probably due to the absence of erythema as a result of the low intensity of the local inflammatory process. These features are associated with the low attraction of neutrophils and macrophages to the sites of inflammation and the subsequent low level of activation of immune mechanisms on the whole. It is possible that the level of IL-8 production (and other attraction substances) is the cause of the development of clinical variants (erythemal or non-erythemal forms) of acute Lyme borreliosis.

The most informative indicators for the differential diagnosis of various forms of Lyme borreliosis are CD3+, IL-8, CD4+ and CD8+; the values of ALC, CD4+/CD8+, PHI, PHN, abs CD3+, IgM, IL-1β and IL-4 were less informative. The relatively low information content of abs CD3+ indicator is a result of its close relationship with ALC, which is a poor informative indicator.

CONCLUSION

Thus, the study of the features of immune status and cytokine production in various forms of Lyme borreliosis identified criteria showing the importance of the fundamental mechanisms of immune regulation in the pathogenesis of tick-borne infections; the expression level of CD3 molecules, the ratios of Th1/Th2 immune response variants, the functional activity of phagocytes and the production of attraction factors were all found to be important.

For differential diagnosis and the comparison of features of the pathogenesis mechanisms of different forms of Lyme borreliosis, the conventional indicators of immune status are informative: CD3+, CD4+ and CD8+. The production of IL-8 indicates the level of intensity of local inflammatory reactions and the attraction of neutrophils and macrophages into sites of inflammation. Therefore, IL-8 is important and necessary as a clinical practice criterion for differential diagnosis and monitoring of patients with various forms of Lyme borreliosis.

These results suggest that the regulation of the immune system in Lyme borreliosis is complex and multidimensional. The features of cytokine regulation of the immune response in different clinical forms of acute Borrelia infection were identified. In different periods of erythemal and non-erythemal forms of Lyme borreliosis, differences in the levels of cytokine production cannot only be a consequence of separate failures of immune defence mechanisms, but also form the basis for clinical manifestations and disease course. The results obtained represent the important precondition for further evaluation of the effectiveness of aetiological treatment and the development of differentiated approaches to immunomodulation.

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