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Analysis of main T-cell subsets and activated T supressor/cytotoxic cells in patients with *Borrelia burgdorferi* s. lato only infection and co-infections with *Anaplasma phagocytophilum*, *Bartonella spp.* and *Babesia microti*

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Abstract

Introduction. The study was designed to assess the role of some important immunologic factors with regards to both laboratory results and clinical symptoms in patients with confirmed Lyme disease. Additional examinations were carried out for co-infections with a number of tick-borne pathogens.

Material and methods. The **s**tudy group consisted of 54 patients with Lyme disease and a group of 21 healthy controls. Serology of co-infections with *Anaplasma phagocytophilum*, *Bartonella spp. and Babesia microti* was carrieed out in all patients. Blood samples were stained using the whole-blood lysis method and analyzed concurrently on a flow cytometer FACSCalibur. Directly conjugated anti-human monoclonal antibodies against CD3, CD4, CD8, CD16, CD56, HLA-DR and CD69 were used.

Results. No significant differences were observed with respect to thepretreatment level of CD4+ and CD8+ cells. In patients with symptoms relief and symptoms persistence, lower percentages of CD4+ and CD8+ cells were found, but with no statistical dependence. In the study group, both in patients with and without co-infections, pretreatment values of CD16+CD56+ cells did not differ significantly. In patients who did not respond to the treatment, the baseline percentage of NK cells was higher ($P<0.01$) than in group with clinical improvement, and lower after the treatment, whereas in patients with symptoms relief after the treatment there was an increase in the percentage of NK cells.

Conclusion. Co-infections with *Anaplasma phagocytophilum*, *Bartonella spp. and Babesia microti* had no impact on T-cell percentages in Lyme disease patients. There was *a* lower baseline percentage of NK cells in patients not responding to antibiotic treatment.

Key words

Lyme disease, *Anaplasma phagocytophilum, Bartonella, Babesia microti*, immunology, T-cell subsets, Therapy

INTRODUCTION

Lyme disease is considered one of the most mysterious and controversial infectious diseases, and is undeniably an infection with significant potential morbidity and increasing prevalence. Most of clinical features are non-typical, and erythema migrans is probably the only one that justifies clinical diagnosis, especially if related to recent tick bite. Joint involvement and neurologic symptoms are not characteristic and broad differential diagnosis is required. The prognosis after antibiotic treatment is generally good, and the efficacy of early therapy has been proved [1]. Nevertheless, some patients may present residual post-treatment symptoms that are difficult to explain. Altogether, clinical manifestations and both questions of firm diagnosis and effective late treatment result in many controversies surrounding Lyme disease. The main problem probably is the lack of particular knowledge and understanding of the pathogenic mechanisms of the disease. Most results make scientists approaching the conclusion consider interaction between spirochetes and host organism to play the key role [2].

Another topic – the role of co-infections acquired from ticks – has been discussed recently in some studies; however, instead of answering difficult questions and drawing scientifically sound conclusions, it has given rise to more controversies. Bearing these challenging problems in mind, the authors decided to carry out theirr own observations in the hope of gaining knowledge important for both doctors and patients [3].

The study was designed to assess the role of some important immunologic factors regarding both laboratory results and clinical symptoms in patients with confirmed Lyme disease. Examinations for co-infections with a number of tick-borne pathogens were also conducted..

Ticks have been implicated as a source of diseases for more than 100 years. The transmission of *Babesia* infections, the causative agent of Texas cattle fever, was described in 1893, many years before the first reports of *Borrelia burgdorferi*

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isolation from *Ixodes* ticks and the recognition this pathogen as the cause of Lyme disease [3, 4]. It has to be admitted that the number of documented human cases of babesiosis was low in the pre-Lyme disease era. During the last 40 years, human infections with different *Babesia* species have been reported [5, 6].

In 1994, human anaplasmosis, previously known as human granulocytic ehrlichiosis, was first reported among patients from Minnesota and Wisconsin in the USA [7]. Since that time, cases of *Anaplasma phagocytophilum* have been described in both Americas and in Europe. The authors of the presented study have previously demonstrated both the co-existence of *Borrelia burgdorferi* sensu lato and *Anaplasma phagocytophilum* in ticks, and seropositivity for both pathogens in inhabitants of Eastern Poland [8].

Some controversies have arisen around the transmission of *Bartonella* spp. by ticks [9]. According to recent studies, the main proof for possible transmission is the presence of *Bartonella* spp. in *Ixodes* spp. ticks collected at various locations in the USA and Europe; however, in only one study the culture was also positive for *Bartonella* spp. [10, 11].

Double or multiple microorganisms coinfections have been previously reported; however, their influence on clinical manifestations and outcomes are not fully understood [3, 7, 8, 10]. Moreover, to the best of the authors' knowledge, there is no complex report of immunologic reactions in patients infected with different tick-borne pathogens. From the practical point of view, the majority of clinicians, particularly general practitioners, who are first-line doctors, have no or very limited experience in both the diagnosis and treatment of them. There is also a risk that other tick-borne illnesses may be mistaken for Lyme disease.

OBJECTIVE

The aim of the presented study was to assess the number of T-cell subsets in patients with Lyme disease before antibiotic therapy for the disease, and 4 months after the first day of antibiotic treatment. Apart from this analysis, the authors wanted to establish the relationship between the immune system interactions to the symptoms, and co-infections with *Anaplasma phagocytophilum*, *Bartonella spp. and Babesia microti*.

MATERIALS AND METHOD

Study populations. The control group comprised 21 healthy volunteers aged 22–25 years: 10 female and 11 male students, without any known pathology, with negative history of any important medical condition, including tick-borne infections and any tick bite.

The study group consisted of 54 patients with the diagnosis of chronic Lyme disease. As there are many diagnostic controversies, the final diagnosis was made based on epidemiologic and clinical data and borrelial serology, according to recommendations and criteria published by Centers for Diseases Control and Prevention (CDC) in Atlanta, USA, and the European Union Concerted Action on Lyme Borreliosis (EUCALB) in Vienna, Austria).

In all patients, both clinical symptoms (however non-specific) and positive serology were observed. Positive reactions in screening ELISA test (Anti-Borrelia IgM, IgG, Euroimmun, Germany) were confirmed with Western-blot test (EuroLine Borrelia IgM, IgG, Euroimmun, Germany). The proper selection of study participants was considered as a key-point; therefore, an attempt was made to exclude all patients in whom the diagnosis of chronic Lyme disease was doubtful or controversial. Predominant symptoms in patients were related to musculo-skeletal involvement, mainly symptoms of migratory polyarthirits or chronic monoarthritis. In 5 patients, neuroborreliosis was diagnosed, with posititive cerebro-spinal fluid test confirmation (Anti-Borrelia IgM, IgG CSF, Euroimmun, Germany); however, all of them also had symptoms of arthritis. As the number of neurologic patients was very small, it was decided not to analyse results in relation to type of symptoms or organ involvement. Patients were treated with a daily dose of 2 g. Ceftriaxone given intravenously. In all patients, blood was taken before the first dose of Ceftriaxone and 4 months later (the second examination was attempted 90 days after the end of monthly treatment).

The study group consisted of 37 women and 17 men, aged 28–73 years (average 51.3 ± 11.4 years). All study group results were compared to those in the control group and between 2 measurements.

Flow cytometry. Anti-coagulated (EDTA) samples of blood were stained using the whole blood lysis method and analyzed concurrently on a flow cytometer FACSCalibur (BD Biosciences) using CellQuest software (BD Biosciences). Directly conjugated anti-human monoclonal antibodies against CD3, CD4, CD8, CD16, CD56, HLA-DR, and CD69 were used. Irrelevant, directly conjugated, mouse anti-human monoclonal antibodies were used to define background staining. All monoclonal antibodies were obtained from BD Biosciences (San Jose, CA, USA) and used as recommended by the manufacturer. Lymphocytes were identified by forward and side scatter. In the case of every cycle of measurements flow, the cytometer was calibrated using CaliBRiTE beads (BD).

To assess each T-cell subset, appropriate kits were used designed to measure the combination of immunophenotypes:

a) CD4 (T helper/inducer) – Simultest CD3/CD4 and Simultest Control γ_1/γ_{2a} (IgG_1 FITC/IgG_2 PE);
b) CD8 (T supressor/cytotoxic) – Simultest CD3/CD8 and Simultest Control γ_1/γ_{2a} (IgG_1 FITC/IgG_2 PE);
c) NK cells (natural killer) CD3/CD16/CD56 – Simultest CD3/CD16 + CD56 and Simultest Control γ_1/γ_{2a} (IgG_1 FITC/IgG_2 PE);
d) activated CD8/CD38 – Simultest CD8/CD38 and Simultest Control γ_1/γ_{2a} (IgG_1 FITC/IgG_2 PE);
e) activated CD8/HLA-DR – Simultest CD8/Anti-HLA-DR and Simultest Control γ_1/γ_1 (IgG_1 FITC/IgG_1 PE);
f) activated CD8/CD69 – FastImmune CD8 FITC/CD69 PE/CD3 PerCP and FAST IMMUNE Control γ1FITC/γ1PE/CD3 PerCP.

Statistical analysis. In statistical analysis, the non-parametric Mann-Whitney test, t-Student test, paired Wilcoxon test and linear regression test were used. All values with $p \leq 0.05$ were considered statistically significant.

RESULTS

Table 1. T-cell subsets in control group

T-cell subset	Peripheral blood lymphocytes ($x_{\pm}$SD)		
	Women (N=13)	Men (N=8)	Total
CD4+ (%)	41.6±7.8	43.2±8.2	42.1±7.9
CD8+ (%)	26.2±6.1	26.9±6.3	26.4±6.3
CD56+/CD16+ (%)	11.4±7.1	10.1±7.3	10.9±7.3
CD8+/CD38+ (%)	7.97±4.1	8.08±4.8	8.01±4.6
CD8+/HLA-DR+(%)	5.27±2.7	5.01±2.99	5.20±2.9

Table 2. T-cell subsets in patients due to coinfection

T-cell subset	Peripheral blood lymphocytes ($x_{\pm}$SD)			
	No coinfection		Coinfection	
	Before treatment	After treatment	Before treatment	After treatment
CD4+ (%)	44.1±10.1	39.5±10.9	43.6±9.1	37.0±8.1
CD8+ (%)	23.2±9.4	23.5±10.9	19.8±7.8	23.4±10.1
CD56+/CD16+ (%)	8.3±5.8	7.7±6.6	7.9±6.5	7.6±5.9
CD8+/CD38+ (%)	16.7±6.9	18.0±6.2	14.3±7.2	16.6±6.2
CD8+/HLA-DR+(%)	9.4±7.7	5.0±3.9	8.2±6.6	7.3 ±6.2

In the group of patients with Lyme disease no statistically significant differences were observed when activated CD8+ cells (with expression of CD38 and/or HLA_DR) before and after treatment were compared. On the other hand, the percentage of activated CD8+ T-cells in the study group was significantly higher than in healthy controls.

The percentage of CD8+CD38+ cells was analyzed in the sub-population of patients with no co-infection and patients with at least one additional tick-borne pathogen seropositivity. No significant differences were found between both group of patients. When CD8+HLA-DR+ cells were analyzed, significantly lower values were observed after the antibiotic treatment in the group of patients with no co-infections ($p<0.01$).

Table 3. T-cell subsets in patients due to symptoms persistence

T-cell subset	Peripheral blood lymphocytes (x ± SD)			
	Symptoms persisted (SP)		Symptoms relief (SR)	
	Before treatment	After treatment	Before treatment	After treatment
CD4+ (%)	39.0 ± 6.2	32.8 ± 7.8	46.1±9.8	39.4±10.1
CD8+ (%)	18.2 ± 8.9	20.3 ± 10.1	22.9±8.5	25.1±8.8
CD56+/CD16+ (%)	6.6 ± 7.0	5.5 ± 3.7	9.5±4.8	8.9±6.9
CD8+/CD38+ (%)	12.0 ± 6.7	17.8 ± 7.7	17.8±6.8	17.9±7.0
CD8+/HLA-DR+(%)	6.3 ± 6.0*#	8.5 ± 5.5#	10.1±5.2*$	5.0±4.9$

* statistical significance SP versus SR
# statistical significance before versus after treatment
$ statistical significance before versus after treatment

While searching for predicting factors of effective therapy of Lyme disease, the percentages of activated CD8+ cells were compared. The baseline percentages of CD8+ cells were similar in patients with effective treatment (clinical symptoms relief). A high standard of deviation was the reason for the failure to prove any statistical significance, although there was a tendency to a lower number of cells with persistent symptoms. Interestingly, in those patients, the percentages of activated T supressor/cytotoxic cells with phenotype CD8+CD38+ were lower.

No significant differences were observed with respect to the pretreatment level of CD4+ and CD8+ cells; nevertheless, there was a tendency toan increase of CD4+ and decline in CD8+ percentages in all analyzed subgroupws of patients when compared to healthy subjects. When analyzing both groups – with symptoms relief and symptoms persistence – both lower percentages of CD4+ and CD8+ cells were found, but again, no statistical dependence was proved.

The most interesting results were noted in natural killer cells (NK), identified as CD16+CD56+ cells. In analysis of the study group, both in patients with and without co-infections pretreatment values and those after the therapy, did not differ significantly. However, in patients who did not respond to antibiotic treatment, the baseline percentage of NK cells was significantly higher ($P<0.01$) than in group with clinical improvement, and lower after the treatment, whereas in patients with symptomps relief, the second examination (after antibiotic treatment) showed an increase in the percentage of NK cells.

DISCUSSION

After analyzing all the clinical signs and symptoms of chronic *Borrelia burgdorferi* infections, it may be concluded that the most characteristic manifestation is the lack of specific clinical features. This situation may be related to the complex form of the pathogen that expresses immunogenic and pro-inflammatory lipoproteins on its surface and, as a consequence, both innate and adaptive immune reactions. In chronic Lyme disease, dissemination of spirochete infection was observed; however, a vigorous inflammatory response that served as the basis of most system and/or organ involvement, is rather related to the host immune response than to direct tissue damage caused by spirochetes [2, 12].

In any infectious disease the immune response depends on the state of the individual's immune system and the type of pathogen. The balance between Th1 and Th2 response could also be altered by some additional factors, such as allergies or atopy, or additional active viral infections (EBV, CMV, etc) that may facilitate Th2 predominance [13, 14]. Immunologic reactions in Lyme disease have been the subject of the number of studies and it has been hypothesized that different Th1 and Th2 predominances of the immune system may have influence on the disease manifestations [12].

In the acute phase of infection, strong initial pro-inflammatory response to *B. burgdorferi* seems to be important for the eradication of spirochetes, and is partly responsible for the development of signs and symptoms of infection. In the case of chronic Lyme disease, the immunopathology is much more complicated [12, 15].

It has been published that the severity of Lyme arthritis is dependent on whether a predominant TH1 or TH2 response is evoked; however, these observation are taken from an animal model [16]. Gross et al. concluded that Th1 predominant response is associated with more severe arthritis symptoms, and generally a Th1 response is responsible for chronic Lyme

symptomatic arthritis [17]. However, recent studies suggest a Th2 predominance in many cases of chronic Lyme disease [18, 19].

The presented study analyzed the proportion of basic lymphocyte subsets, including CD4+, CD8+, NK cells and activated CD8+ cells. They represent non-specific cell-mediated response and, as proved in many infections, may be much more important in both pathogenesis of clinical symptoms and clearing the infection. No significant differences were found in the current study in subsets of CD4+ and CD8+ between patients with B. burgdorferi mono-infection and co-infections. The study also shows the post-treatment decline of the percentages of activated CD8+ cells, but only in patients without any co-infection. This may suggest that the presence (current or past) of another tick-borne pathogen may make the treatment of Lyme disease more difficult.

Due to the small number of patients with a different tick-borne pathogen additional to *B. burgdorferi* infection, it was decided to assess patients with any coinfection as one group. This may be a limitation of the study analysis. But there are more such limitations, including false-positive and false-negative reactions, and possible cross-reactivity between tick-borne pathogens, such as *A. phagocytophilum* and *B. burgdorferi,* which should be considered in interpreting the epidemiologic conclusions of the presented study [20, 21]. The discussion about the results obtained is difficult, because no prospective studies have been conducted to assess the immunologic effects on humans. Experimental studies on animals have revealed that simultaneous infection with *B. burgdorferi* and *A. phagocytophilum* modulates the immune response and affects the development of arthritis. In a mouse model, co-infection increased the number of $CD4^+$ cells and drove cytokine release toward a T helper 1 (Th1) lymphocyte response. Co-infection with *B. burgdorferi* and *B. microti* has also been demonstrated to have immunologic effects in animal models, including alteration of the Th1 cell response and increased severity of arthritis [22]. In another mouse model, dual infection with *B. burgdorferi* and *B. microti* appeared to follow independent courses [23].

Patients simultaneously infected with *B. burgdorferi* and *B. microti* appear to have more diverse, intense, and persistent symptoms. In a prospective study, co-infected patients were twice as likely to report influenza-like symptoms than patients with Lyme disease alone; they also had a higher incidence of splenomegaly [24, 25].

The current observations and results from a number of animal models are controversial. Great care should be taken in drawing any conclusion, especially bearing in mind the increasing interest of patients in this problem demanding aggressive treatment, and the lack of well-controlled clinical studies.

In the presented study, a significantly lower percentage of NK cells was found in patients who did not respond to antibiotic treatment. This observation is extremely important due to a recent hypothesis that the CD57 NK cell subset serves as a predicting factor for the development of chronic Lyme disease. Stricker et al., using a flow cytometry test system with an established normal range and well-defined coefficient of variation, found that the $CD3^-$ $C57^+$ NK subset appeared to be a useful immunologic marker in patients with persistent Lyme disease symptoms, compared to either normal subjects or disease control [26]. However, the conclusions of their results differ from the findings of the study by Marques et al., and these controversies were the point of discussion between the authors [27, 28]

It must be borne in mind that the measurement of $CD3^-$/$CD57^+$ cells is not a standard flow cytometry approach for the measurement of natural killer (NK) cells [26, 27]. In the current study, the routine approach for NK quantitation was used, with a combination of CD56 and CD16 surface expression, together with negative staining for CD3 (to exclude T cells expressing NK markers).

When considering the lower baseline percentage of NK cells in patients not responding to standard antibiotic treatment, one has to be very cautious with possible explanations. First, the lack of clinical improvement must not to be related with active borrelial infection because most symptoms are of a subjective nature. Second, many of the symptoms resemble chronic fatigue syndrome (CFS). In 2007, Michaylova et al. have proved that the expression of the CD69 activation marker on T cells (CD3+, CD3+CD4+, and CD3+CD8+) and on NK cells (CD45+CD56+), was significantly lower in CFS patients than in healthy subjects. The authors concluded that patients with CFS showed defects in T- and NK cell activation [28]. Therefore, is *Borrelia burgdorferi* infection a possible reason for chronic fatigue syndrome?

There are some suggestions for future study that might explain more precisely the mechanisms of mixed infections with different tick-borne pathogens. Prospective studies should involve healthy persons with frequent exposure to tick bites. Research is also needed to understand the role of genetic heterogeneity among coinfecting pathogens. Further efforts are needed to assess immune response for true infections, excluding the role of other factors, including the role of antimicrobial medications that are often overused in Lyme disease patients.

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