

Concentration of TGF- β 1 in the supernatant of peripheral blood mononuclear cells cultures from patients with early disseminated and chronic Lyme borreliosis

Grygorczuk S^{1*}, Chmielewski T², Zajkowska J¹, Świerzbinska R¹, Pancewicz S¹, Kondrusik M¹, Tylewska-Wierzbanowska S², Hermanowska-Szpakowicz T¹

¹ Department of Infectious Diseases and Neuroinfections of the Medical University of Białystok, Poland

² Department of Chlamydiae, Rickettsiae and Zoonotic Spirochetes of the National Institute of Hygiene, Białystok, Poland

Abstract

Purpose: The aberrant inflammatory response is probably involved in the pathogenesis of chronic Lyme borreliosis, including chronic Lyme arthritis and neuroborreliosis. Transforming growth factor-beta 1 (TGF- β 1) is an important anti-inflammatory and immunomodulatory cytokine and its deficient synthesis is linked to exaggerated inflammation and immune response.

Material and methods: Peripheral blood mononuclear cells (PBMC) from 25 patients with Lyme borreliosis and 6 controls were incubated for 7 days with suspension of *Borrelia afzelii*, *B. garinii* and *B. burgdorferi* sensu stricto spirochetes. TGF- β 1 concentration in culture supernatants was measured with ELISA. Results were analyzed according to disease duration (group I – chronic borreliosis, n=20; group II – early borreliosis, n=5) and clinical form (LA – arthritis, NB – neuroborreliosis).

Results: TGF- β 1 concentration was increased in supernatants of PBMC cultures of patients with early neuroborreliosis, in comparison with chronic borreliosis and controls. In chronic, but not in early borreliosis, there was a tendency for decrease of TGF- β 1 synthesis under stimulation with *B. burgdorferi* spirochetes.

Conclusions: Impaired synthesis of TGF- β 1 by mononuclear cells seems to be present in patients with chronic forms of Lyme borreliosis when compared to those with early stage of the disease. It may be a factor contributing to the persistence of inadequate inflammatory response in patients in whom chronic form of the disease develops.

Key words: Lyme borreliosis, arthritis, neuroborreliosis, inflammation, transforming growth factor-beta1.

Introduction

Lyme borreliosis is a chronic and multiform infectious disease caused by a spirochete *Borrelia burgdorferi* sensu lato (*B. burgdorferi* s.l.) transmitted to humans from animal reservoir by *Ixodes* sp. tick. Three clinical stages: localized, early disseminated and chronic infection are distinguished in the course of the disease, with lesions typically developing within skin (erythema migrans, acrodermatitis chronica atrophicans), musculoskeletal system (Lyme arthritis) and central and peripheral nervous system (neuroborreliosis) [1,2]. Arthritis symptoms range from brief attacks of arthralgia, through intermittent episodes of arthritis to chronic arthritis and/or persistent musculoskeletal pain. Central nervous system involvement may manifest itself early as comparatively mild meningitis or in chronic form as a progressive encephalopathy or leukoencephalitis, the later presenting with focal demyelination and some clinical resemblance to sclerosis multiplex [2,3]. Response to antibiotic therapy tends to be good in early and more variable and generally poorer in later stage of the disease, with some patients exhibiting treatment-resistant symptoms, pathogenesis of which has not been fully explained [2,3]. *B. burgdorferi* s.l. expresses surface lipoproteins with potent pro-inflammatory properties, however, it does neither produce toxins nor damage the tissues directly, and spirochete number within affected tissue is relatively low with regard to the severity of the lesions. In some patients with chronic Lyme arthritis symptoms persist even after *B. burgdorferi* s.l. DNA becomes undetectable within the lesions [4]. Development of the chronic, antibiotic-resistant form of the disease is associated with certain types of major histocompatibility complex (MHC) molecules and some features of humoral and cellular response against *B. burgdorferi* s.l., especially with strong response to OspA lipoprotein [4-6].

* CORRESPONDING AUTHOR:

Department of Infectious Diseases and Neuroinfections
Medical University of Białystok
ul. Żurawia 14, 15-540 Białystok, Poland
Tel: +48 085 7409519, -514, Fax: +48 085 7409515
e-mail: neuroin@amb.edu.pl (Sambor Grygorczuk)

Received 14.11.2006 Accepted 06.04.2007

These observations led to assumption that it is not active infection, but rather inability to control inflammatory and immune reaction against *B. burgdorferi* s.l., or even autoimmunity triggered by the infection, which contribute to the pathogenesis of chronic Lyme arthritis, and possibly also chronic neuroborreliosis [2,5-8]. However, the mechanisms of autoimmunity remain enigmatic, and the role of proposed self-antigens has not been confirmed [5].

TGF- β 1 is a pleiotropic cytokine with a wide range of anti-inflammatory and immunosuppressive effects, antagonistic towards a wide array of pro-inflammatory cytokines. It plays unique role in ensuring control and proper termination of inflammatory reaction, and its deficient synthesis has been observed in autoimmune disorders [9-14]. As such, TGF- β 1 impaired synthesis could be possibly involved in the pathogenesis of the chronic Lyme borreliosis, but so far data on its role in this disease has been few.

The inflammatory infiltrates in Lyme borreliosis are typically mononuclear, with presence of lymphocytes specifically recognizing *B. burgdorferi* s.l., and the generalized character of the infection is reflected by the changes in peripheral blood lymphocyte subpopulations as well [8,15-17]. In this study, we used peripheral blood mononuclear cells (PBMC) as an indicator of immune and inflammatory processes undergoing in the disease lesion, measuring concentrations of TGF- β 1 in the supernatant of PBMC cultures from patients with early disseminated as well as chronic Lyme borreliosis, incubated with *B. burgdorferi* s.l. spirochetes, with an aim of detecting a possible impairment of TGF- β 1 synthesis.

Material and methods

Patients

The study group consisted of 25 patients with Lyme borreliosis (6 women and 19 men) aged from 24 to 62 years ($\bar{x}\pm SD=46.6\pm 9.2$), hospitalized in years 2004-2005 in the Department of Infectious Diseases and Neuroinfections of the Medical University of Białystok. Patients were qualified for the study according to diagnosis made by a treating physician and documented in a medical record. All patients reported tick bites or frequent exposure to bites in endemic areas and had clinical symptoms suggestive of Lyme borreliosis. All patients had antibodies against *B. burgdorferi* s.l. detected in serum. Basic laboratory tests (ESR, leukocyte count) showed no signs of acute inflammatory response. Dependent on clinical features, other examinations (rheumatoid factor detection and other serological tests, radiological examination of the joints) were performed to exclude other possible etiologies. Lyme arthritis (LA) was diagnosed in patients with musculoskeletal pain, especially affecting the large joints of the extremities. Neuroborreliosis (NB) was diagnosed on the basis of the abnormalities in the neurological examination, inflammatory changes in cerebrospinal fluid (CSF) examination and presence of antibodies against *Borrelia burgdorferi* s.l. in the CSF. Twenty patients with the history of complaints persisting or recurring for more than 6 months (in 18 patients – more than a year, maximum – 8 years) constituted the group of chronic borreliosis (group

I) – 4 women and 16 men, mean age – 48.4 ± 8.4 years. They all had been at least once treated with doxycycline and/or III generation cephalosporin, typically with a temporary improvement, after which ailments recurred. Early disseminated borreliosis was diagnosed in 5 patients (2 women and 3 men, aged 39.8 ± 10.1) with symptoms present for less than 6 months and anti-*B. burgdorferi* s.l. IgM class antibodies detectable in serum (group II). Diagnosis of LA was established in 21 patients: 19 from group I and 2 from group II, and NB in 9 patients, including 6 in the group I and 3 in the group II (5 patients, all from the group I, had both symptoms of LA and neuroborreliosis). The control group consisted of 6 patients (3 women and 3 men, aged 41.7 ± 11.6), without suspicion of Lyme borreliosis or after it was excluded, all without detectable antibodies against *B. burgdorferi* s.l. in serum and without any features of acute inflammatory response. All participants gave a conscious consent and a study design was approved by the Ethical Committee of the Medical University of Białystok.

Detection of anti-*B. burgdorferi* s.l. antibodies

Antibodies against *Borrelia burgdorferi* s.l. were detected in a sample taken on EDTA with ELISA, using a kit from Biomedica (Boston, USA), according to manufacturer's instructions. The kit included panel of following recombinant antigens: *B. burgdorferi* s.l. p21 (OspC), *Borrelia garinii* p41 and *Borrelia afzelii* p41 to detect IgM and p21, *B. garinii* p41, *B. afzelii* p41, p18 and p100 to detect IgG. The results were expressed in BBU/ml (Biomedica *Borrelia* units/ml), with >11 BBU/ml considered as positive. The same diagnostic kit was used for antibody detection in CSF.

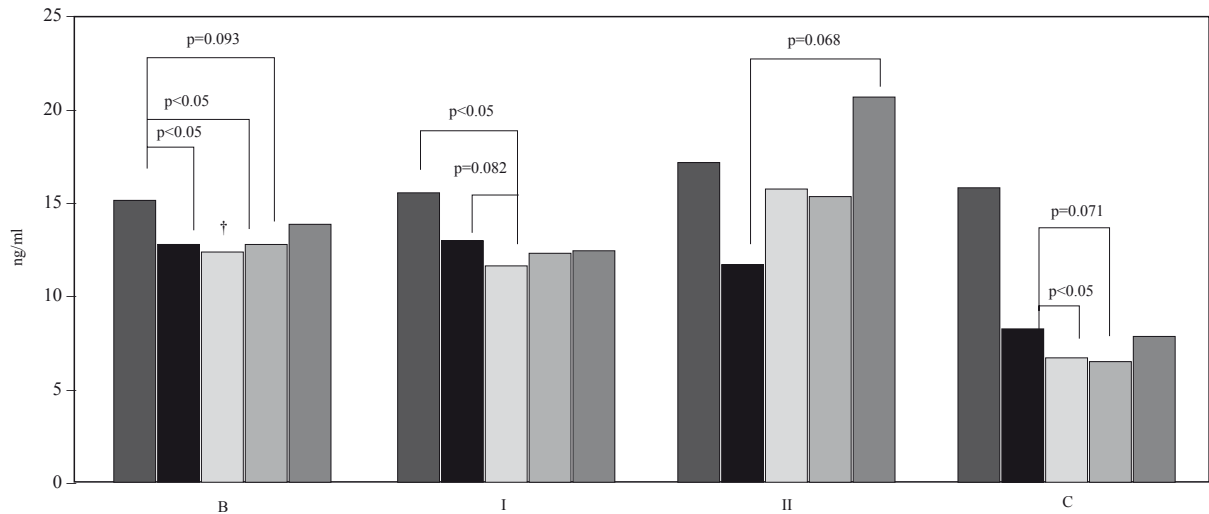
Cell culture

The samples were obtained before the start or during the first week of antibiotic therapy. Ten ml of venous blood was taken to heparinised tubes and further processed within 30 min. PBMC were isolated by centrifugation in Gradisol G medium (Aqua Medica, Poland) at 400 g for 30 min. After centrifugation PBMC were suspended in the culture medium RPMI 1640 (Biomed, Poland) and rinsed again. Then they were resuspended in the concentration of 2×10^6 cells/mL in RPMI 1640 with addition of 10% inactivated bovine serum, streptomycin and penicillin. The suspension of 10^5 cells/well was incubated in the 24-well plate Nunclon Multidishes (Nunc Brand Products, Denmark), in 5% CO₂, at 37°C, with the culture medium alone as a negative control, culture medium and phytohemagglutinin (Biomed, Poland) as a positive control and suspension of spirochetes *B. afzelii* VS 461 (ATCC 51567) (B.a.), *B. garinii* 20047 (ATCC 51383) (B.g.) and *B. burgdorferi* s.s. B31 (ATCC 35210) (B.ss.) at the concentration of 10^8 cells/well. Culture conditions and time were based on our previous experiments and selected to enable maximum PBMC response to antigenic stimulation [16]. After 7-day incubation, the culture was centrifuged and the supernatant was frozen and stored in -70°C until being tested.

TGF- β 1 detection

In the supernatant, TGF- β 1 concentration was determined by ELISA, using the kit from Bender MedSystems (Vienna,

Figure 1. Mean concentration of TGF- β 1 in the supernatant of the culture of PBMC from patients with Lyme borreliosis and from controls. B – patients with borreliosis in total (n=25), I – patients with chronic borreliosis (n=20), II – patients with early disseminated borreliosis (n=5), C – controls (n=6). Concentrations were determined in the supernatant obtained after 7-day culture. Bars from the left to the right in each group, represent, respectively: no stimulation, stimulation with phytohemagglutinine and stimulation with antigens of *Borrelia afzelii*, *Borrelia garinii* and *Borrelia burgdorferi sensu stricto* (see: Material and methods). Significant differences between the cultures within the groups are marked on the chart. † – borderline difference (p=0.079) in comparison with controls



Austria), following the manufacturer's instructions. The detection limit of the test according to the manufacturer was 0.06 ng/ml and the standard curve covered the concentration range from 0.47 to 30 ng/ml.

Statistical analysis

Results were analyzed in the group of patients with Lyme borreliosis as a whole and in the subgroups defined on the basis of the duration of the disease (chronic borreliosis – I, early – II) and clinical form (LA and NB). There were only two patients with early LA, so this group was not included in the analysis. LA and NB, as well as chronic LA and chronic NB groups were not compared directly with one another, since they overlapped due to coexistence of articular and neurological symptoms in some patients. Analysis was performed with SSST software. The Mann-Whitney test was used to compare groups. Comparisons between the cultures within groups were performed using t test for dependent samples. $p < 0.05$ was regarded as significant and $p < 0.1$ as a borderline significance.

Results

Results for the group of patients with Lyme borreliosis as a whole, group I, II and controls, with statistical interpretation, are presented in Fig. 1. Analogous data for neuroborreliosis patients are shown in Fig. 2. The results for patients with LA and chronic LA did not differ from those found in group I and are not presented separately.

The mean concentration of TGF- β 1 in the supernatant tended to decrease in the cultures stimulated with phytohemagglutinin in comparison with unstimulated ones. In the antigen-stimulated cultures in controls and group I, there was a tendency for TGF- β 1 concentration decrease to values lower even than at phytohemagglutinin stimulation. In the NB group and in group

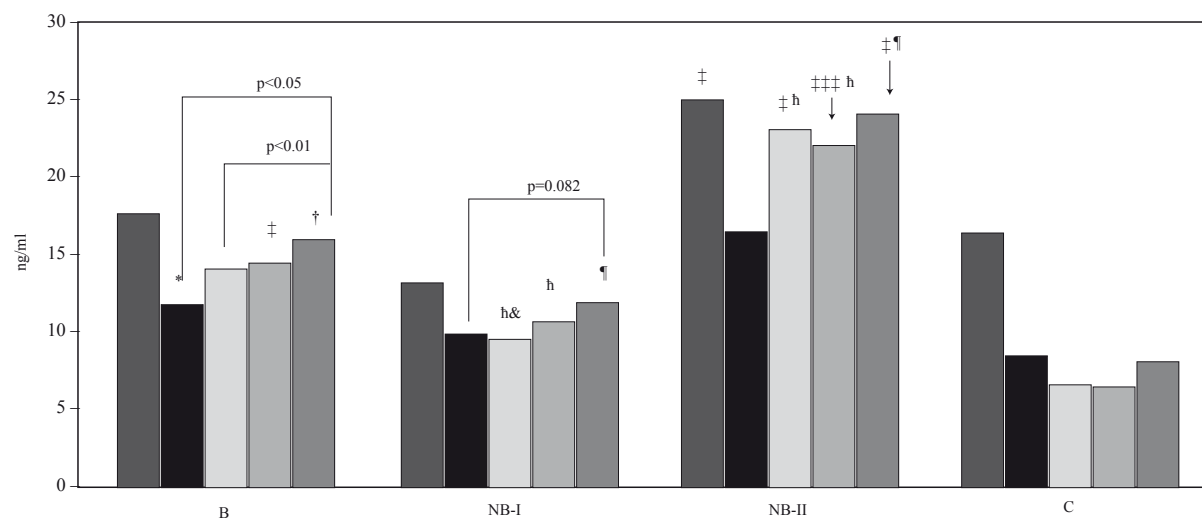
II, such a tendency was not present, and concentrations in the antigen-stimulated cultures tended to be higher than with the phytohemagglutinin stimulation, which was statistically significant for B.ss. stimulation.

TGF- β 1 concentrations showed a tendency to increase in patients with borreliosis in comparison with controls, however the difference was statistically significant only in the NB group, especially in early neuroborreliosis patients (NB-II), in which TGF- β 1 concentrations were significantly higher than in controls both under antigenic stimulation and without stimulation. In NB-II the concentration of TGF- β 1 in antigen-stimulated cultures was also significantly or with borderline significance higher than in chronic neuroborreliosis (NB-I) and Lyme arthritis groups (LA).

Discussion

TGF- β 1 is a potent anti-inflammatory cytokine, released by most of the cell types, especially by leukocyte populations, including activated macrophages and lymphocytes [18,19]. It is released in latent form, which requires further activation to exert its biologic effects. Increased synthesis of TGF- β 1 in the final stages of inflammatory and immune response establishes anti-inflammatory local environment and promotes tissue repair, by stimulating release of other anti-inflammatory factors, counteracting pro-inflammatory cytokines, inhibiting functions of B and T lymphocytes and finally stimulating local fibrosis and angiogenesis [11,14,18,19]. In animal models of inflammation and autoimmunity, TGF- β 1 appears to be a factor essential for termination of the inflammation and resolution of inflammatory infiltrate, both in Th1 and Th2-dependent type of inflammatory response [9,10,13]. Administration of TGF- β 1 has been shown to prevent or ameliorate symptoms and histological features of inflammation in animal model of sclerosis

Figure 2. Mean concentration of TGF- β 1 in the supernatant of the culture of PBMC from patients with neuroborreliosis and from controls. NB – patients with neuroborreliosis in total (n=9), NB-I – patients with chronic neuroborreliosis (n=6), NB-II – patients with early neuroborreliosis (n=3), C – controls (n=6). Concentrations were determined in the supernatant taken after 7-day culture. Bars from the left to the right within each group represent, respectively: no stimulation, stimulation with phytohemagglutinine and stimulation with antigens of *Borrelia afzelii*, *Borrelia garinii* and *Borrelia burgdorferi* sensu stricte (see: Material and methods). & – borderline difference in comparison with lack of stimulation ($p=0.068$), * – significant difference in comparison with lack of stimulation ($p<0.05$), other differences between cultures are marked directly on the chart; † – borderline difference ($p=0.086$) in comparison with controls; ‡ – significant difference in comparison with controls, $p<0.05$; ‡‡‡ – highly significant difference in comparison with controls, $p<0.001$; ¶ – borderline difference between patients with early and chronic neuroborreliosis, $p=0.059$; h – significant difference between patients with early and chronic neuroborreliosis, $p<0.05$



multiplex, as well as in acute and chronic arthritis [9,20,21]. Mokhtarian et al. observed that peripheral blood lymphocytes from patients with sclerosis multiplex synthesize decreased levels of TGF- β in culture, which coincided with increased synthesis of pro-inflammatory cytokines [12]. Moreover, the defective production of TGF- β correlated with clinical activity of the disease [12]. As the inability to properly control and terminate inflammatory response seems to underlie pathogenesis of chronic Lyme borreliosis, impaired synthesis of TGF- β 1 might be of importance in this group of patients as well.

To our knowledge, the only study assessing the TGF- β 1 in human Lyme borreliosis was conducted by Widhe et al., who measured serum concentrations of TGF- β 1 in patients with erythema migrans (early, localized stage of Lyme borreliosis) and in the early and late stage of neuroborreliosis. The authors monitored the patients for several months and checked for correlation of the early TGF- β 1 levels with further clinical course of the disease. The serum concentration of TGF- β 1 in early borreliosis appeared to be higher in patients with a favorable course leading to recovery in comparison with those in whom borreliosis finally developed into the chronic stage [21]. In the same study, analogous observation was made for cerebrospinal fluid concentration of tumor necrosis factor- α (TNF- α), a strong pro-inflammatory factor. According to the authors, the synthesis of TNF- α in the early stage of the disease is a sign of a potent inflammatory response leading to eradication of spirochetes, while a high level of TGF- β 1 is a marker of the effectiveness of an anti-inflammatory reaction, preventing prolonged inflammation and further tissue damage [22]. High levels of TGF- β 1 were also observed in the sera of patients with erythema migrans and persisted for several months after successful

treatment, which supports the importance of this immunoregulatory factor in recovery from Lyme borreliosis [22].

In our previous studies we assessed concentration of transforming growth factor- β 1 (TGF- β 1) in sera of patients with different clinical forms of Lyme borreliosis, observing weakly increased TGF- β 1 concentration in Lyme arthritis after antibiotic treatment in comparison with controls [23]. No change in TGF- β 1 concentration was observed in patients with erythema migrans or neuroborreliosis. Afterwards, we measured TGF- β 1 concentration in the supernatant of *B. burgdorferi* s.l. antigens-stimulated culture of peripheral blood mononuclear cells (PBMC) obtained from a small group of 10 patients with chronic Lyme arthritis, finding no significant differences in comparison with unstimulated cultures and cultures of PBMC from healthy persons [24].

In our present study, concentrations of TGF- β 1 in the PBMC culture supernatant from patients with chronic, treatment resistant Lyme borreliosis, tended to decrease in presence of *B. burgdorferi* s.l. spirochetes. This phenomenon suggests development of pro-inflammatory environment in response to borrelial antigens in this group of patients. In patients with early infection concentration of TGF- β 1 did not decrease in the presence of *B. burgdorferi* s.l. antigens and was generally higher in comparison with patients with chronic borreliosis and controls. This group of patients was small and not all clinical forms of Lyme borreliosis were equally represented, especially we were not able to include sufficient number of patients with early Lyme arthritis into the study, which makes the results more difficult to interpret, however, some differences seem apparent. The increase of TGF- β 1 under stimulation with *B. burgdorferi* s.l. in early neuroborreliosis, statistically significant in spite of

a small number of patients in this group, is especially of note. It is paralleled by the clinical difference between this subacute, antibiotic-responding form of Lyme borreliosis, and late, poorly responding to treatment manifestations present in other patients, in whom analogous TGF- β 1 response was apparently absent. This observations are in accordance with the interpretation of serum measurements in erythema migrans and neuroborreliosis as presented by Widhe et al. [22]. This finding could be further confirmed by studying TGF- β 1 synthesis by mononuclear cells directly isolated from the disease lesion, especially from synovial fluid or synovium of patients with Lyme arthritis.

The results suggest a shift of the balance towards the pro-inflammatory response in patients with chronic, treatment resistant Lyme borreliosis. An ability to control an inflammatory and immune response against *Borrelia burgdorferi* s.l. spirochetes, present in an early stage, may be impaired in patients in whom Lyme borreliosis changed to a chronic form and impaired synthesis of TGF- β 1 by patients' mononuclear cells in response to *B. burgdorferi* s.l. seems to be involved in the pathogenesis of this phenomenon.

Acknowledgments

This study was funded by the Medical Academy of Białystok, grant no 3-45651L.

References:

1. Åsbrink E, Homark A. Comments on the course and classification of Lyme borreliosis. *Scand J Infect Dis*, 1991; 77: (Suppl.): 41-3.
2. Steere AC, Coburn J, Glickstein L. The emergence of Lyme disease. *J Clin Invest*, 2004; 113: 1093-101.
3. Logigian EL, Kaplan RF, Steere AC. Chronic neurologic manifestations of Lyme disease. *N Engl J Med*, 1990; 323: 1438-44.
4. Carlson D, Hernandez J, Bloom BJ, Coburn J, Aversa JM, Steere AC. Lack of *Borrelia burgdorferi* DNA in synovial samples from patients with antibiotic treatment-resistant Lyme arthritis. *Arthritis Rheum*, 1999; 42: 2705-9.
5. Steere AC, Falk B, Drouin EE, Baxter-Lowe LA, Hammer J, Nepom GT. Binding of outer surface protein A and human lymphocyte function-associated antigen 1 peptides to HLA-DR molecules associated with antibiotic treatment-resistant Lyme arthritis. *Arthritis Rheum*, 2003; 48: 534-40.
6. Chen LZ, Hochwald GM, Huang C, Dakin G, Tao H, Cheng C, Simmons WJ, Dranoff G, Thorbecke GJ. Gene therapy in allergic encephalomyelitis using myelin basic protein-specific T cells engineered to express latent transforming growth factor-beta1. *Proc Natl Acad Sci USA*, 1998; 13: 12516-21.
7. Kalish RA, Leong JM, Steere AC. Association of treatment-resistant chronic Lyme arthritis with HLA-DR4 and antibody reactivity to OspA and OspB of *Borrelia burgdorferi*. *Infect Immun*, 1993; 61: 2774-9.
8. Steere AC, Duray PH, Butcher EC. Spirochetal antigens and lymphoid cell surface markers in Lyme synovitis. Comparison with rheumatoid synovium and tonsillar lymphoid tissue. *Arthritis Rheum*, 1988; 31: 487-95.
9. Sigal LH. Cross-reactivity between *Borrelia burgdorferi* flagellin and a human axonal 64,000 molecular weight protein. *J Infect Dis*, 1993; 167: 1372-8.
10. Hansen G, McIntyre JJ, Yeung P, Berry G, Thorbecke GJ, Chen L, DeKruyff RH, Umetsu DT. CD4+ T helper cells engineered to produce latent TGF- β 1 reverse allergen-induced airway hyperactivity and inflammation. *J Clin Invest*, 2000; 105: 61-70.
11. Kehrl JH, Roberts AB, Wakefield LM, Jakowlew S, Sporn MB, Fauci AS. Transforming growth factor β is an important immunomodulatory protein for human B lymphocytes. *J Immunol*, 1986; 137: 3855-60.
12. Mokhtarian F, Shi Y, Shirazian D, Morgante L, Miller A, Grob D. Defective production of anti-inflammatory cytokine, TGF- β by T cell lines of patients with acute multiple sclerosis. *J Immunol*, 1994; 152: 6003-10.
13. Thorbecke GJ, Umetsu DT, deKruyff RH, Hansen G, Chen LZ, Hochwald GM. When engineered to produce latent TGF-beta1, antigen specific T cells down regulate Th1 cell-mediated autoimmune and Th2 cell-mediated allergic inflammatory processes. *Cytokine Growth Factor Rev*, 2000; 11: 89-96.
14. Wahl SM, Hunt DA, Wong HL, Dougherty S, McCartney-Francis N, Wahl LM, Ellingsworth L, Schmidt JA, Hall G, Roberts AB, Sporn MB. Transforming growth factor- β is a potent immunosuppressive agent that inhibits IL-1-dependent lymphocyte proliferation. *J Immunol*, 1988; 140: 3026-32.
15. Steere AC, Coburn J, Glickstein L. The emergence of Lyme disease. *J Clin Invest*, 2004; 113: 1093-101.
16. Zajkowska JM, Badania nad znaczeniem mechanizmów immunologicznych u chorych z boreliozą z Lyme. Praca habilitacyjna, Białystok; 2002
17. Zajkowska J, Hermanowska-Szpakowicz T, Wysocka J, Kondrusik M, Pancewicz S, Grygorczuk S. Analysis of some peripheral blood lymphocyte subsets in relation to *Borrelia burgdorferi* antibodies in patients with Lyme disease. *Rocz Akad Med Białymst*, 2000; 45: 184-98.
18. Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF- β , PGE2 and PAF. *J Clin Invest*, 1998; 101: 890-8.
19. McDonald PP, Fadok VA, Bratton D, Henson PM. Transcriptional and translational regulation of inflammatory mediator production by endogenous TGF- β in macrophages that have ingested apoptotic cells. *J Immunol*, 1999; 163: 6164-72.
20. Kuruvilla AP, Shah R, Hochwald GM, Liggitt HD, Palladino MA, Thorbecke GJ. Protective effect of transforming growth factor β 1 on experimental autoimmune diseases in mice. *Proc Natl Acad Sci USA*, 1991; 88: 2918-21.
21. Brandes ME, Allen JB, Ogawa Y, Wahl SM. Transforming growth factor β 1 suppresses acute and chronic arthritis in experimental animals. *J Clin Invest*, 1991; 87: 1108-13.
22. Widhe M, Grusell M, Ekerfelt C, Vrethem M, Forsbergh P, Ernerudh J. Cytokines in Lyme borreliosis: lack of early tumor necrosis factor- α and transforming growth factor- β 1 responses are associated with chronic neuroborreliosis. *Immunology*, 2002; 107: 46-55.
23. Grygorczuk S, Pancewicz S, Kondrusik M, Świerzbńska R, Zajkowska J, Hermanowska-Szpakowicz T. Apoptosis in Lyme borreliosis – a preliminary study. *Med Sci Monit*, 2003; 9: CR449-55.
24. Grygorczuk S, Zajkowska J, Świerzbńska R, Pancewicz S, Kondrusik M, Hermanowska-Szpakowicz T. [Concentrations of soluble factors participating in regulation of apoptosis of lymphocytes from patients with chronic Lyme arthritis (preliminary report)]. *Pol Merk Lek*, 2006; 20: 49-52.