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Evaluation of Th1/Th2 lymphocyte balance and lypopolysaccharide receptor expression in asthma patients

Abstract

Introduction: An increase in the number of asthma patients which has recently been observed depends on their place of residence and their occupation. This suggests that both external factors and genetic predispositions affect the development of the disease. The contact with bacterial lypopolysaccharide (LPS) may suppress the development of asthma among rural inhabitants. The mechanism of LPS effect most probably consists in the activation of macrophages and granulocytes by TLR4 and CD14 receptors for the production of cytokines, which affect Th1/Th2 balance.

The objective of the study was the evaluation of CD14 and TLR4 expression on mononuclear cells and the analysis of Th1/Th2 balance in peripheral blood among asthma patients.

Material and methods: The study group covered 22 patients with bronchial asthma (mean age 45 ± 15), and was conducted by the method of flow cytometry with the use of fluorochrome-labelled monoclonal antibodies. CD14 and TLR expression was assessed in peripheral blood monocytes. Th1/Th2 balance was determined by the measurement of intracellular IL-2, IFN- γ , IL-4 and IL-10 expression in T-helper cells after culture with the stimulation of cytokine production.

Results: A negative correlation was noted between TLR4 expression and the percentage of Th2 lymphocytes, while a positive correlation was observed between expression of TLR4 and percentage of Th1 cells. No relationship was found between CD14 expression on monocytes and the percentage of Th1 and Th2 lymphocytes.

Conclusions: An increased percentage of lymphocytes with TLR4 expression is associated with the change in Th1/Th2 balance in favour of Th1 lymphocytes in asthma patients.

Key words: asthma, Th1/Th2 balance, LPS, TLR4, CD1

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Introduction

Asthma is a chronic inflammatory disease of the respiratory system [1, 2]. Typical clinical symptoms are shortness of breath, coughing, wheezing and chest tightness. These symptoms are accompanied by bronchial hyperactivity. In the course of the disease there occur periods of aggravation and remission [3]. The drugs of choice are inhaled glucosteroids, the main effect of which is the attenuation of the inflammatory response [4]. Asthma is a disease of varied etiology, which has not been fully recognised. In the complex pathomechanisms

of asthma there participate many cells of the immune system, together with the substances secreted by these cells [5, 6]. The forms of asthma on allergic and non-allergic backgrounds may be distinguished.

T-helper lymphocytes perform an important function in asthma mechanism (Th, CD4+ T-helper lymphocytes) [7–9]. There are two main subpopulations of T-helper lymphocytes: Th1 and Th2. The primary role of Th1 lymphocytes is their participation in the cellular response, among other things, in the course of viral, protozoon infections, as well as cancer. The typical substan-

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ces produced by Th1 lymphocytes are interleukin 2 (IL-2) and gamma interferon (IFN- γ). Th1 lymphocytes also exert an inhibitory effect on allergic reactions. Th2 lymphocytes play a crucial role in responses of the humoral type, favouring the development of allergy. The characteristic compounds produced by Th2 lymphocytes are interleukin 4 (IL-4) and interleukin 10 (IL-10) [10]. Among patients with allergic diseases the Th1/Th2 balance is in favour of Th2.

It is believed that asthma affects 300 million people worldwide [11]. The results of epidemiological studies conducted in Poland indicate that this disease afflicts 4.8–5.4% of the Polish population, and the mortality is 2 cases per 100,000 inhabitants [12–14]. An upward tendency in asthma morbidity is observed [11]. A large increase in the number of asthma patients suggests that the cause is due more to external factors than to genetic predisposal.

Many scientists started their research by trying to determine factors predisposing to asthma. A 1989 study showed a significantly smaller occurrence of allergies among children from families with several children, as an expression of a greater exposure to infections [15]. This study became the foundations for a 'hygienic theory' of asthma development, according to which smaller exposure to microorganisms in the early childhood causes the transition of Th1/Th2 balance towards Th2, therefore towards the development of allergy. Comprehensive studies were conducted which indicated that the occurrence of allergy, and at the same time of asthma, was lower among children from agricultural regions, compared to urban children [16–18]. A similar situation was noted among adults [19]. A significantly elevated level of endotoxin was observed in rural as opposed to urban households, as well as a lower prevalence of bronchial asthma among children exposed to endotoxin [20, 21].

Toxins secreted by bacteria may be divided into endotoxins and exotoxins. Endotoxins are bacterial cell wall fragments. In Gram-negative bacteria this is lypopolysaccharide (LPS) [22]. LPS has a strong immunomodulatory effect, inducing the response on the part of B, T lymphocytes, macrophages, mast cells and dendritic cells. LPS expressed by bacteria binds to a soluble form of receptor for LPS — CD14 (sCD14) [23]. This phenomenon takes place due to LPS binding protein — LPB. The LPS-sCD14 complex is transported to the CD14 receptor present on the membranes of neutrophils and macrophages (membrane bound CD14 — mCD14). LPS-mCD14 complex binds to TLR 4 in the presence of MD-2 protein, which causes the transmission of a signal to the cell and the beginning of

the production and expression of integrins, TNF and interleukins, through the activation of, among other things, transcription factor NF- κ B.

TLR receptors (Toll-like receptors) participate in many immune processes [22]. Most receptors are present on the surface of cells and possess in the extracellular part leucine-rich domains, whereas the domains in the intracellular part are identical, as in IL-1 receptors (which possess Toll-IL-1R domain — TIR). In this numerous group, TLR4 receptor may be distinguished, which is the receptor for LPS and lypoteichoic acid. This receptor occurs, among others, on the epithelial cells of the airways, endothelium, and on leukocytes. Epithelial cells of the airways stimulated by lypopolysaccharide secrete chemokines, defensins and cytokines, the aim of which is the activation and attraction of cells of the immune system. Mast cells, via signals from TLR4 receptors on their surface, produce many mediators (histamine, PAF, LTB₄, PGD₂), aimed at strengthening the signals informing the immune system of infection (chemotaxis, stimulation of lymphocytes and macrophages, decrease in the permeability of the vessels). Attracted and preliminarily activated macrophages and lymphocytes, which also possess on their surface receptors for LPS, secrete many substances in order to control bacterial infection.

The objective of this study was assessment of CD14 and TLR4 expression on mononuclear cells, and analysis of Th1/Th2 balance by determination of the intracellular IL-2, IFN- γ , IL-4 and IL-10 expression in peripheral blood T-helper lymphocytes in asthma patients.

Material and methods

Characteristics of the patients in the study and preparation of peripheral blood

The study covered 22 patients with asthma (mean age 45 ± 15): 13 patients with atopic asthma, and nine with non-atopic asthma, who reported for planned check-up examination to the outpatient pulmonology clinic of the Pneumonology, Oncology and Allergology Department. During the last months the patients did not show any features of asthma exacerbation requiring antibiotic therapy and treatment with oral glucocorticosteroids. The patients chronically applied inhaled steroids and broncholytics, while not being administered other drugs acting on the function of the immune system.

For immune tests, 10 ml of peripheral blood was taken from the patients into heparinised sy-

ringes. During the centrifugation of the peripheral blood ($700 \times g$, 20 min.) in density gradient of Lymphoprep preparation (Nycomed, Norway) mononuclear cells were isolated. Subsequently, the cells in interphase were collected and washed with physiological salt buffer solution without Ca^{2+} and Mg^{2+} (PBS, Biomed, Lublin).

Assessment of receptor expression for LPS: CD14 and TLR4 on mononuclear cells

A part of the isolated cells in the amount of 1×10^6 per tube were incubated for 20 minutes at room temperature with the set of the following monoclonal antibodies:

- anti-IgG1 FITC and anti-IgG2a PE (Becton Dickinson, USA) — negative control;
- anti-CD14 FITC (Becton Dickinson, USA), and anti-TLR4 PE (Bio Scene, USA) — assessment of TLR4 expression on CD14^+ monocytes.

After completion of incubation the cells were washed twice in PBS and instantly analysed by flow cytometry.

Determination of intracellular expression of cytokines in Th lymphocytes

The remaining cells were grown in 24-hour cell cultures (37°C , 5% CO_2) on RPMI media, with the addition of 10% fetal bovine serum (PAA, Austria), and a set of antibiotics (1% v/v penicillin/streptomycin, Sigma, Germany). After 24 hours, phorbol myristate acetate (PMA, 2.5 ng/ml), ionomycin (2 ng/ml) and brefeldin were added directly to the culture wells. After the subsequent five hours, culture cells were collected and washed in PBS without Ca^{2+} and Mg^{2+} .

In order to determine the expression of CD4 antigen, anti-CD4 FITC monoclonal antibody was applied (Becton Dickinson, USA), in which the cells were incubated for 20 minutes at room temperature. For the fixing of marking with antibody and permeabilising cellular membrane, the Intra-Prep kit (Beckman Coulter, USA) was used according to the producer's recommendations. The expression of intracellular cytokines was determined with the use of the following monoclonal antibodies (BD Biosciences, Pharmingen, USA):

- PE-conjugated rat anti-human IL-2;
- PE-conjugated mouse anti-human IL-4;
- PE-conjugated rat anti-human IL-10;
- PE-conjugated mouse anti-human IFN- γ .

In order to evaluate the expression of intracellular cytokines, a flow cytometer was applied (FACSCalibur, Becton Dickinson, USA) equipped with an argon laser emitting radiation bundle at the wavelength of 488 nm, and CellQuest programme.

The differences in the expression of the antigens examined, compared to the isotype control, were confirmed by Kolmogorov-Smirnov test (K-S). The statistical analysis was performed by means of Wilcoxon test and R Spearman correlation test with the use of Statistica 5.0 PL software. The results obtained were presented as mean values $\pm$ standard deviation. The research project was acknowledged by the Bioethical Commission.

Results

In the presented study, CD4-positive cells showing intracellular IL-2 and IFN- γ expression were defined as Th1 helper lymphocytes, whereas CD4-positive cells showing IL-4 and IL-10 intracellular expression were defined as Th2 helper lymphocytes.

The percentage of Th1 lymphocytes producing IL-2 and IFN- γ in the peripheral blood was significantly higher than the percentage of Th2 cells with IL-4 and IL-10 expression. In addition, the percentage of Th1 lymphocytes producing IL-2 was significantly higher than the percentage of cells with IFN- γ expression. The expressions of IL-2 and IFN- γ were significantly higher than the expressions of IL-4 and IL-10, and the expression of IL-10 was significantly higher than IL-4 expression (Fig. 1). All the above-mentioned significant values remained at the level of $p < 0.0001$. No statistically significant differences in the percentages of lymphocytes producing the examined cytokines were observed between atopic and non-atopic patients. Figure 2 presents an example of an image of cytometric analysis of the percentage of lymphocytes with intracellular expression of the cytokines in the study.

The analysis showed that $67.8 \pm 11.6\%$ of peripheral blood lymphocytes possessed a slight (8.83 ± 2.35 MFI) TLR4 expression. However, this was different from the isotype control (K-S test; $p < 0.001$). The percentage of monocytes showing TLR4 expression, however, was $20.8 \pm 25.1\%$. TLR4 expression was significantly higher on peripheral blood monocytes (176.8 ± 82.1 MFI) than on lymphocytes ($p < 0.0001$). The expression of CD14 antigen on peripheral blood monocytes was 1264.2 ± 226.9 MFI, on average. Figures 3 and 4 show the cytometric analysis of TLR4 receptor expression on peripheral blood monocytes and lymphocytes.

Among patients in the study, a significantly negative correlation was noted between the percentage of Th2 lymphocytes with intracellular expression of IL-4 ($R = -0.429$; $p < 0.05$; Fig. 5B), and IL-10 ($R = -0.462$; $p < 0.05$), and the expression of TLR4 on lymphocytes. A significantly negative cor-

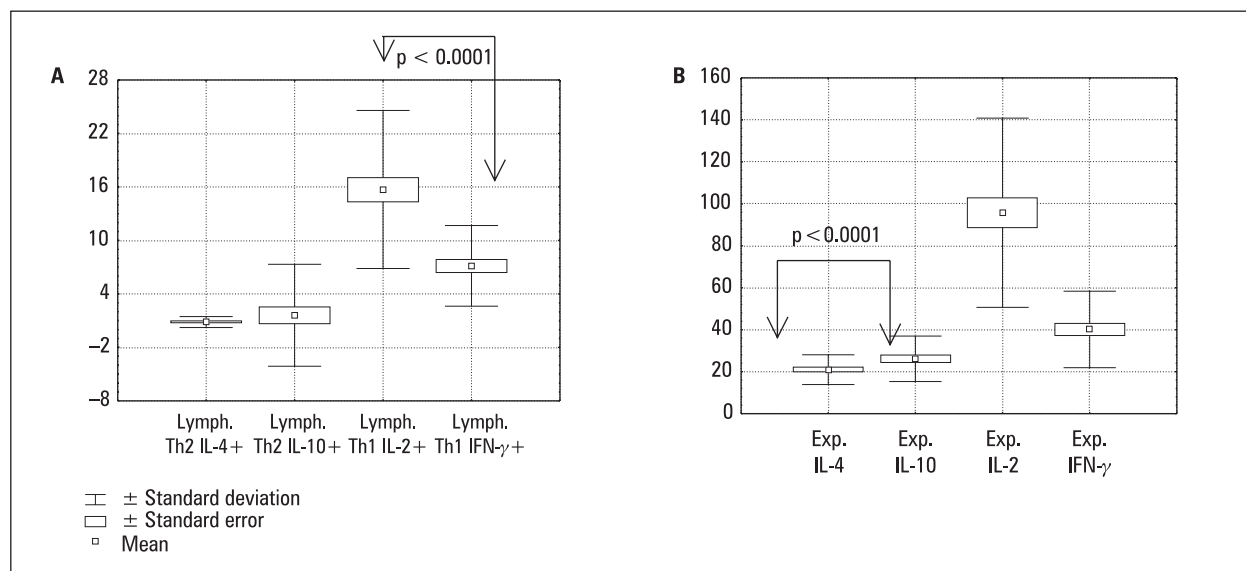


Figure 1. Percentages of Th1 lymphocytes with intracellular expression of different cytokines (A); comparison of intracellular cytokines expression in subpopulation of T-helper lymphocytes (B)

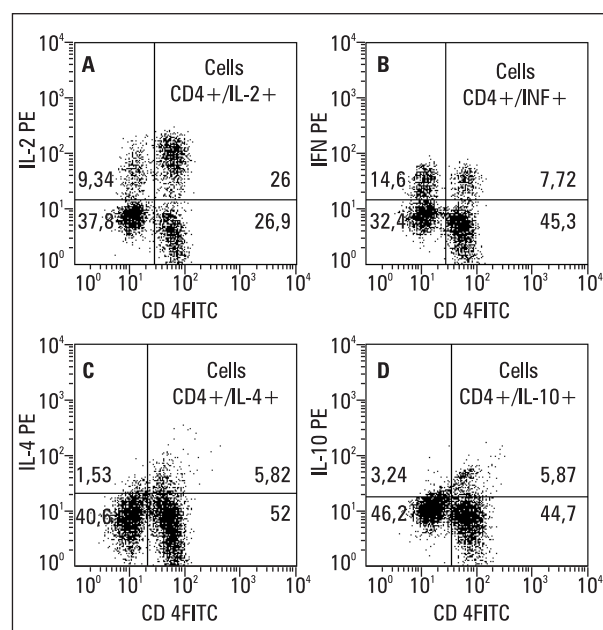


Figure 2. The representative flow cytometry analysis of the percentage of Th lymphocytes with intracellular expression of: IL-2 (A), IFN- γ (B), IL-4 (C) and IL-10 (D)

relation was also observed between the percentage of monocytes showing TLR4 expression, and the percentage of CD4⁺ cells producing IL-4 ($R = -0.44$; $p < 0.05$). The percentage of Th1 lymphocytes with intracellular IL-2 expression, however, positively correlated with TLR4 expression on lymphocytes ($R = 0.404$; $p = 0.06$; Fig. 5A).

No statistically significant correlations were noted between the percentage of Th1 and

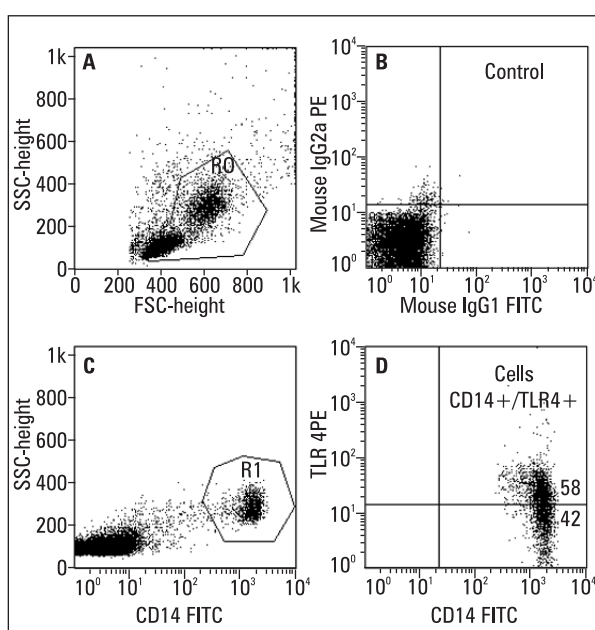


Figure 3. The representative flow cytometry analysis of TLR4 receptor expression on monocytes isolated from peripheral blood. The mononuclear cells from gate R0 were analysed according their SSC parameter and CD14 antigen expression. The expression of TLR4 receptor was estimated only among CD14-positive cells

Th2 lymphocytes with CD14 antigen expression in peripheral blood monocytes. It was only confirmed that the intracellular IL-10 expression in Th2 lymphocytes positively correlated with the expression of CD14 antigen on monocytes ($R = 0.438$; $p < 0.05$).

Discussion

Epidemiological studies conducted among asthma patients did not provide a clear image of the effect of patients' exposure to lipopolysaccha-

ride in the clinical course of the disease [22]. It is known, however, that antigen stimulation of the immune system, including that with bacterial lipopolysaccharide, plays an important role in the working out of basic protective mechanisms in the development of asthma.

A 'hygienic theory' was created, according to which more frequent exposure to infections exerts a protective effect and decreases the risk of asthma, while smaller exposure to bacterial factors is conducive to the transition of Th1/Th2 balance in the direction of Th2. This theory was confirmed by the studies of families of lower socio-economic status and of families with several children, where a rarer occurrence of allergies and bronchial asthma was observed among children [15].

The data from literature suggests that apart from population and environmental studies, which attracted the researchers' attention to this relationship, the effect of TLR4 gene polymorphisms, as well as the CD14 particle expression, was also noted on the capability of lymphocytes participating in allergic response for the production of various cytokines. The occurrence of these polymorphisms may result in the abnormal functioning of the immune processes, thus contributing to the development of atopy or even asthma [24–31]. Fageras et al. observed a relationship between a decreased production of IL-12 and IL-10 by LPS stimulated lymphocytes, and a certain polymorphic form of TLR4 gene, the presence of which positively correlated with the increased incidence of asthma [24]. Based on the results of their studies, Fageras et al. proposed a thesis concerning the weakening of the Th2 pathway and transition of the immune response towards Th1 in children with asthma. As a result of the response to LPS, the cells of the immune system secrete various substances which favour the formation of cytotoxic lymphocytes and Th1

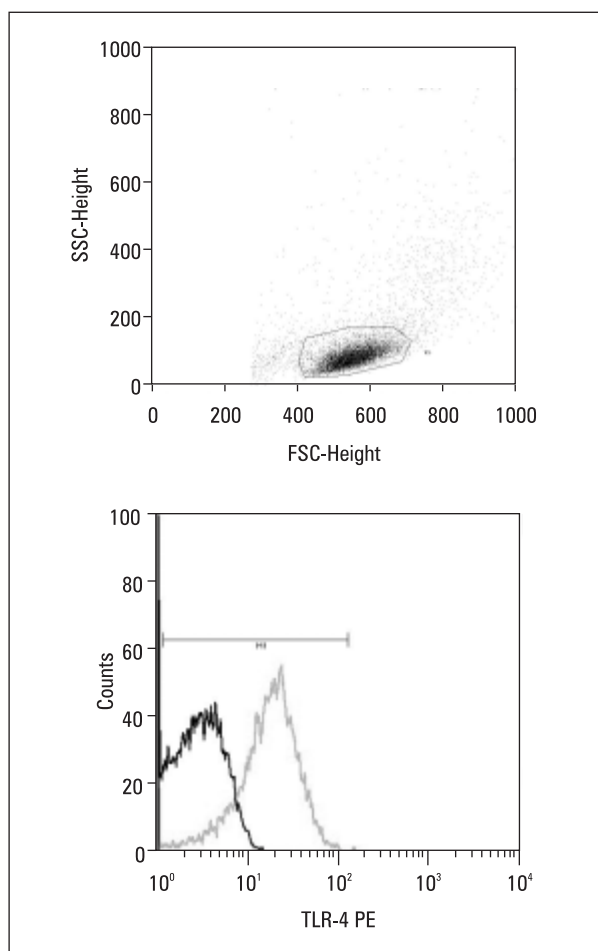


Figure 4. The representative flow cytometry analysis of TLR4 receptor expression on peripheral blood lymphocytes: ■ negative control; ■ examined sample

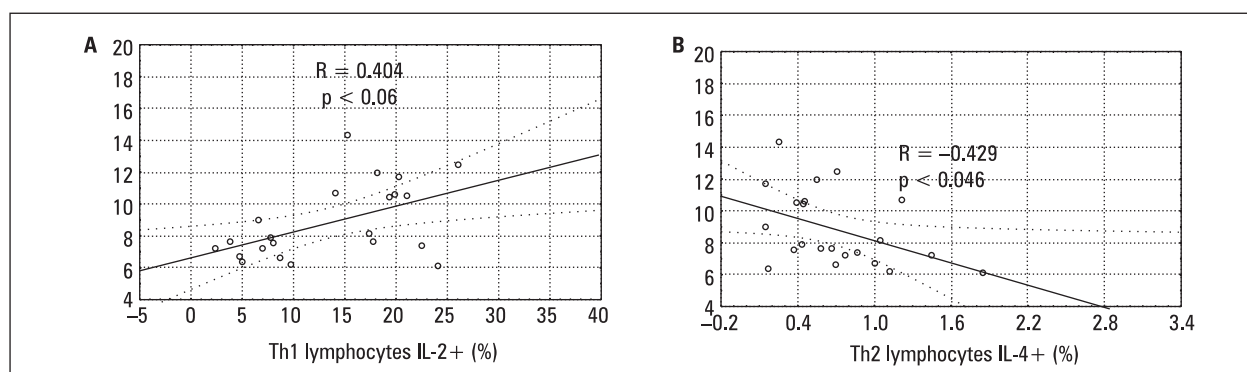


Figure 5. The correlation between the percentage of Th1 lymphocytes with intracellular expression of IL-2 and the expression of TLR4 receptor on lymphocytes (A); the correlation between the percentage of Th2 lymphocytes with intracellular expression of IL-4 and the expression of TLR4 receptor on lymphocytes (B)

cells, inhibition of the formation of IgE by IL-12, and an increase the secretion of IFN- α , IFN- γ [22, 23]. The studies conducted by Koch et al. confirmed a weakened Th1 response following stimulation with lypopolysaccharide in adult patients with asthma [28]. This phenomenon may be explained by a decreased TLR4 expression in CD4+ lymphocytes observed by the researchers in the group of patients examined.

The objective of the study presented was the determination of the relationship between Th1/Th2 balance in asthma and the expression of CD14 antigen and TLR4 receptor on mononuclear cells stimulated with lypopolysaccharide. LPS exerts a non-specific activatory effect on lymphocytes and macrophages, bonding with specific, circulating in blood LBP protein. This protein mediates in the transition of LPS on CD14 receptor, which at the final stage facilitates the LPS interaction with the target receptor — TLR4. By the application of the flow cytometry method, the study noted a positive correlation between the percentage of Th1 helper lymphocytes producing IL-2 or IFN- γ , and the percentage of mononuclear cells showing TLR4 expression. Simultaneously, a negative relationship was observed between the percentage of Th2 lymphocytes and TLR4 expression on mononuclear cells. These results unequivocally indicate that there was a Th1 type of response in the peripheral blood of the group of patients examined, which additionally correlates with an increased TLR4 receptor expression. However, no relationship was noted between CD14 antigen expression and Th1/Th2 balance. Shirai et al. also observed a decreased level of Th2 in peripheral blood among patients with asthma and atopy, compared to the healthy control group [32]. The presented study, however, focused on the determination of Th1/Th2 balance in the group of asthma patients. In light of the studies by Shirai et al. it is recommended that the Th1/Th2 balance should be determined also in the healthy group, and the differences between asthmatic patients and healthy individuals should be determined.

Koch et al. [28] conducted studies on the effect of LPS on the production of various cytokines by lymphocytes. The researchers evaluated Th1/Th2 balance in patients with asthma with respect to the stimulation of cells with various LPS concentrations, and observed decreased Th1 type of response to LPS, manifested by reduction in the production of suitable cytokines. However, LPS did not exert such a significant effect on the population of Th2 cells in the group of patients examined.

In this study, the prevalence of Th1 over Th2 type response was observed, which is probably

associated with an increase in the percentage of mononuclear cells with TLR4 receptor expression, whereas the expression of CD14 antigen was not significantly related with the population of Th1 lymphocytes. TLR4 is the main receptor for lypopolysaccharide. After analysis of the results of the presented study, it seems that not only the exposure to lypopolysaccharide, but also the level of receptors expression for LPS may determine the type of induced immune response in asthma patients. In addition, TLR4 expression on mononuclear cells may correlate with the level of exogenous LPS and affect the Th1/Th2 balance in a way opposite to that of LPS alone.

The level of exposure of the organism to lypopolysaccharide is also of importance. Eisenbarth et al. in their studies of mice, observed that a low level of inhaled lypopolysaccharide caused the activation of Th2 lymphocytes via TLR receptor, with an additional contribution of the group of myeloid dendritic cells [33]. Inhalations of high concentration of LPS, however, stimulated the development of Th1 type of response. The data suggests that LPS level exerts a significant effect on the type of immune response induced.

Many studies conducted on mice models confirmed that not only LPS and TLR expression play an important role in the pathogenesis of asthma (the development of inflammation), but primarily also in the joint effect of antigen and endotoxin [34]. According to animal models, lypopolysaccharide may show various effects on the inflammatory process in the airways. Jung et al. proved that after the administration of antigen there occurred a considerable intensification of the inflammatory process in mice strains with TLR4 expression deficiency. In turn, Hollingsworth et al. evaluated the effect of low doses of LPS and various lengths of exposure of the immune system of mice to antigen [35]. The researchers confirmed that short-time exposure to allergen did not cause differences in immune response in mice with TLR4 deficiency and in the control group, while long-time exposure to allergen resulted in a considerable increase in markers of the active inflammatory process in mice with TLR4 deficiency.

Conclusions

Summarising the results, it appears that Th1 lymphocytes may constructively show the expression of the TLR4 antigen. Based on this assumption, the correlation between TLR4 expression and the lymphocytes' capability for the production of suitable cytokines shown in this study may be

a result of the phenotype possessed by Th1 lymphocytes, and may not be significantly related to the induction of IL-2 and INF- γ production by lypopolysaccharide acting via the TLR4 receptor.

Despite numerous studies of asthma, there has been no entirely successful explanation of the relationship between immunology and the clinical status of a patient. The theory concerning the disturbance of the Th1/Th2 balance in favour of Th2 may be questionable. There are reports which indicate that the prevalence of Th1 lymphocytes results in asthma exacerbation, while an elevated Th2 level, among other things in parasitic invasions, decreases the risk of exacerbation. The search began for the factors which affect the Th1/Th2 regulation. Such factors are regulatory lymphocytes (Treg; CD4⁺, CD25^{high}) which, via regulatory protein FoxP3 and secreted TGF- β , exert an inhibitory effect on Th1 and Th2 lymphocytes. The growing out of allergy was observed in children with a higher Treg level, as well as an increase in Th2 induced by allergen, caused by Treg blockage. Treg may attenuate allergic response to bacterial antigen via dendritic cells [36]. A decreased Treg level was also noted in atopy, especially during the pollen season, reflected by Th1/Th2 balance, or during the aggravation of asthma [37, 38]. The complete mechanisms still require elucidation, and contradictory information may result from the period when blood was taken for tests [36].

Many clinical studies confirm the relationship between the effect of lypopolysaccharide on the development of asthma, and the degree of its intensity. The effect of TLR4 receptor on Th1/Th2 regulation in this disease has not been fully recognised. It seems that this is a very complex process, dependent on many factors, including polymorphism of suitable receptors, type and concentration of LPS, presence of antigen and the length of time of exposure. The data obtained in this study remain associated with the results of studies conducted in many centres dealing with the pathomechanism of bronchial asthma. The results obtained show the complexity of the problem which requires further study, including undertaking the determinations in healthy individuals.

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