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Examination of correlation between vitamin D_3 ($25\text{-}OHD_3$) concentration and percentage of regulatory T lymphocytes (FoxP3) in children with allergy symptoms

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Abstract

Populations of T lymphocytes cells called regulatory T lymphocytes (Tregs) play a key role in the formation and maintenance of immune tolerance. This phenotypically heterogeneous group of cells regulates the immune system and plays an important role in tumor immunology, transplant immunology and the pathogenesis of multiple disorders, in particular autoimmune diseases. In the study it was decided to investigate the difference in the correlation of levels of vitamin D_3 ($25\text{-}OHD_3$) with the percentage of Tregs cells (FoxP3+) in children with no symptoms of allergy (control group), and with symptoms of allergy. In addition, in both groups the phenotype of lymphocytes was identified. The results indicate a positive correlation between Tregs and $25\text{-}OHD_3$ only in children with allergic symptoms (no correlation in the control group), along with a simultaneous significant reduction in the percentage of CD4 lymphocytes in this group.

Key words: *allergy, regulatory lymphocytes, vitamin D, children's diseases.*

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Introduction

The immune tolerance of organism is more than passiveness, ignoring antigens and leaving them alone. It is also active recognition of self antigens by T lymphocytes and issuing orders banning the executive cells for aggressive behavior against their own tissues. Maintaining of this condition is possible due to the interaction of the lymphocyte with antigen in the early stages of its development. Cells that have not acquired tolerance in central lymphoid organs, are deleted or become anergic in peripheral lymphoid organs [1]. A crucial role in the formation and maintenance of immune tolerance is played by the defined populations of lymphocytes T. This phenotypically heterogeneous group of cells regulates the immune system, participates in the control and inhibition of excessive immune responses (especially against their own antigens), play an important role in tumor immunology, transplantation immunology and the pathogenesis of multiple disorders, including autoimmune diseases [2-5].

Natural Tregs (nTregs) constitute 5-10% of peripheral CD4+ cell pool with co-expression of CD25 receptor; their survival and functioning depend on IL-2. The transcription factor FoxP3 is considered a specific nTregs marker, which is crucial for the development of a regulatory cell activity. Constant and high co-expression of CD25 surface antigen differs them from the activated T cells CD4+ CD8+, which have a lower and transient expression of this marker [6-9].

In this heterogeneous population of regulatory lymphocytes (CD4+), besides nTregs there are also sub-pop-

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ulations of induced regulatory T cells, such as Th3, Tr1 cells and anergic cells included. Stimulation of naive CD4+ cells *in vitro* in the presence of exogenous IL-10 leads to the differentiation of Tr1 population which, following antigenic stimulation produces large amounts of IL-10 and is capable of inhibiting the activity of Th1 and Th2 cells *in vivo*. Another stimulator of Tr1 cell differentiation *in vitro* is interferon γ [10-12]. Oral administration of antigen stimulates the activity of another Tregs cell subpopulation – Th3. These cells produce TGF-β (transforming growth factor β), immunosuppressive cytokine and a "switch" for the synthesis of IgA. The final population of Tregs cells are anergic T cells which have the ability to inhibit T cell responses *in vitro* and *in vivo* through a mechanism that modify the function of antigen presenting cells (APC) [6, 12, 13].

In recent years, studies were published showing a relationship between the concentration of metabolites of vitamin D and the incidence of autoimmune diseases such as diabetes mellitus type 1 and multiple sclerosis [14-17]. Gathered evidence confirmed that vitamin D might prevent the development of these diseases, by increasing the number or the effect of the naturally occurring Tregs cells [15, 16].

In the world literature, there are few reports about the relationship between vitamin D levels and the amount of regulatory T-cell subpopulations. This study aimed to examine the correlation of vitamin D_3 (25-OHD_3) with the percentage of Tregs lymphocytes (FoxP3+) in children with allergic symptoms. The studies are preliminary and will be the basis to conduct further experiments that expand our knowledge in this field.

Material and methods

The study was conducted on a group of 19 children who were treated from January to April 2012 in the Paediatric, Nephrology and Allergology Clinic, Military Institute of Medicine in Warsaw. In all examined children there was a tendency to airway obstruction confirmed by clinical trial and in some cases by the spirometry test. In the same time, the concentration of 25-OHD_3 in the serum was determined in all patients and the information concerning diet, supply of vitamins and living conditions including sun exposure were collected during the interview. In all children the profile of immune cells (CD19, CD4, CD8, CD25/FoxP3), and NK cells (CD16/56) were determined.

Based on interview information, clinical symptoms, level of immunoglobulin E (IgE) in the blood serum and the results of skin prick tests (SPT) (made in some patients), children were divided into two groups: a control group (without clinical signs of allergy, with low IgE level) and a group with clinical symptoms of atopic allergy (with elevated IgE level, diagnosed asthma and/or atopic dermatitis). Characteristics of these groups of patients are shown in Table 1; all values are expressed as mean (± SE). There were no statistical differences between the groups referred to characteristics.

25-hydroxyvitamin D_3 (25-OHD_3) and IgE were determined in serum samples by commercially available enzyme immunoassay kits according to the manufacturer's instructions.

Regulatory T cell percentages were determined by surface and intracytoplasmic staining peripheral blood mononuclear cells with fluorochrome-coniugated mouse antibodies against: CD4-PerCP, CD25-APC and FoxP3-PE (BD Biosciences), according to the manufacturer's protocol. For examination of lymphocyte subsets, samples were labeled with antibodies against: CD3, CD4, CD8, CD19 and CD16/56 (IMK Test, BD Biosciences). The samples were analyzed by flow cytometry using appropriate isotype controls. Cytometry analyses were performed using FACS Calibur Flow Cytometer equipped with CellQuest Software (BD Biosciences).

The obtained results are presented as mean values and standard errors. Nature of the data distribution was checked using the Shapiro-Wilk test. For comparison between separate groups, *t* test was used if the distribution of the collected data was a normal distribution and the homogeneity of variance was maintained. In other cases – the Mann-Whitney *U* test were used. In order to investigate the dependence of the parameters (concentration of 25-OHD_3 and the percentage of regulatory cells) analysis were performed using the Pearson correlation statistical method.

The significance level was $p < 0.05$.

The study is part of a research project which was approved by the Bioethics Committee WIL 108/12.

Table 1. Characteristics of tested groups of children (Average number or mean ± SE)

	Control group	**Group with symptoms of atopic allergy**
Average age (years)	4.37 ±1.19	4.18 ±1.28
Number of boys	8	6
Number of girls	1	4
Average number of leukocytes (×10^9/l)	6.89 ±0.59	8.30 ±1.32
Average percentage of lymphocytes (%)	37.90 ±5.29	45.60 ±5.05
Average percentage of monocytes (%)	6.15 ±0.90	7.12 ±0.60
Average percentage of neutrophils (%)	49.09 ±6.10	40.21 ±5.21
Average percentage of eosinophils (%)	3.45 ±1.80	2.15 ±0.58
Average percentage of basophils (%)	0.29 ±0.11	0.69 ±0.15

Results

In the obtained results there was significantly lower mean concentration of total IgE in serum of children with no symptoms of allergy (control group) in comparison to allergic group (Table 2). Although in the control group mean concentration of this immunoglobulin was tenfold lower, the difference was not statistically significant. This is probably due to the large differences between individuals (especially in the group with symptoms of allergy), and a small, highly diverse in terms of coexisting diseases in control group. Similarly obserwation was found with the average concentration of vitamin D_3 (25-OHD_3). However the differences were not so significant and the lower concentrations was found in the control group (Table 2).

In the study subpopulation of regulatory T cells was defined as: CD4+, CD25++ and nuclear factor FoxP3+ expression. To define this, the cells in the population of mononuclear cells were firstly visualized for CD4 surface marker (Gate 1, R1). Next within this population (G1) were determined cells with high expression of CD25 and expression of intracellular nuclear factor FoxP3 (Gate 2, R2). Typical cytogram from the investigation is shown in Fig. 1, and the average number and percentages of regulatory cells in the blood of the children presented in Table 3.

In this study we also define the size of main subpopulations of lymphocytes. Average percentages of B cells, T and NK cells (CD19+, CD4+, CD8+ and CD16/56+) are shown in Fig. 2. There was a statistically significant decrease in CD4 lymphocytes in children with the recognized allergy compared to the control group.

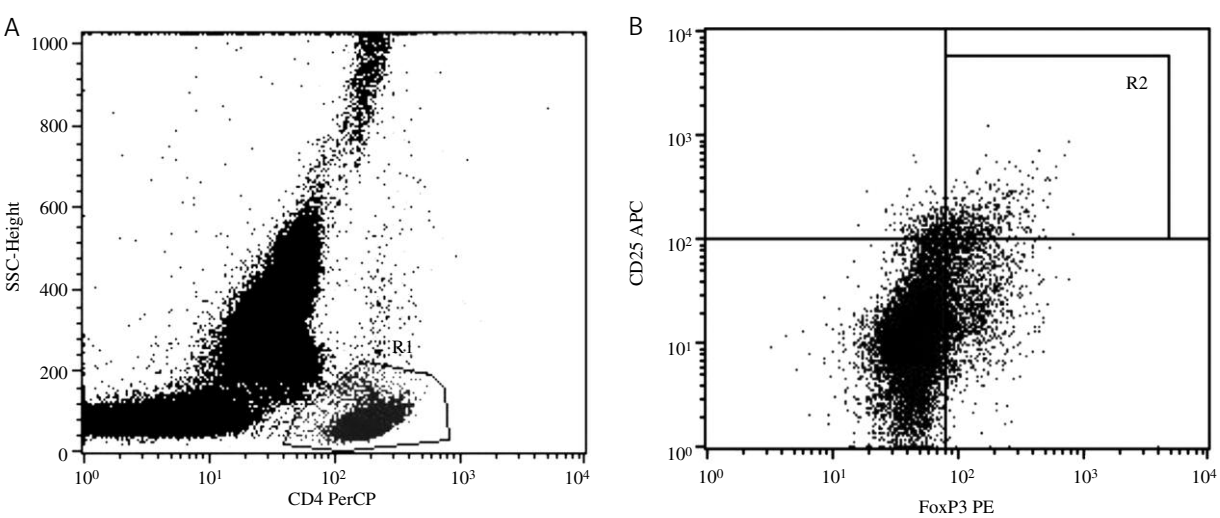
In a further step of the research a relation between the concentration of 25-OHD_3 and percentage of regulatory cells (CD4+, CD25+ FoxP3+) was analyzed. The results are shown in Fig. 3 – for children with clinically recognized allergy and in Fig. 4 for the control group. The obtained data show that there is a positive correlation between Tregs cells and 25-OHD_3 only in children with allergic symptoms. The correlation factor was 0.772 in this case, a significance

Table 2. The average level (± SE) of IgE and 25-OHD_3 in the serum of the children

	Control group	Group with symptoms of atopic allergy
Average level of total IgE (IU/ml)	51.56 ±11.99	698 ±389.59
Average concentrations of vitamin 25-OHD_3 (ng/ml)	20.54 ±3.28	18.63 ±3.20

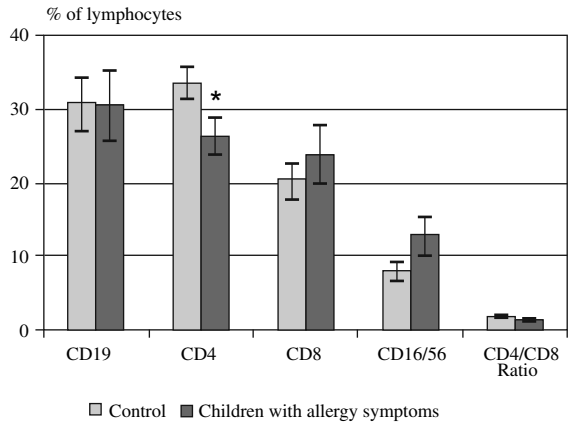
Table 3. Average number and percentages of regulatory cells in the blood of the children ± SE

	Control group	Group with symptoms of atopic allergy
Average number of regulatory T cells in blood (×10^6/l)	35.31 ±10.41	25.88 ±7.16
Average percentage of regulatory T cells among leukocytes (%)	0.50 ±0.12	0.35 ±0.09
Average percentage of regulatory T cells among CD4+ lymphocytes (%)	4.14 ±1.37	3.67 ±0.69



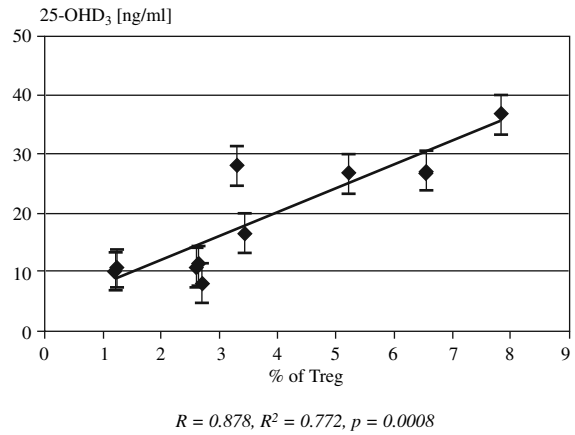
R1 – a separate sub-population of CD4+, R2 – lymphocyte subpopulation of regulatory CD4+/CD25+/FoxP3+

Fig. 1. Typical cytogram of one of the patients showing the labeling percentage of regulatory cells among the CD4+ lymphocyte subsets



**Differences are statistically significant (when compared to an appropriate control group) at the level of $p < 0.05$*

Fig. 2. Average percentages (± SE) of the main subpopulations of lymphocytes in the blood of children identified with no allergy symptoms (Control) and symptoms of allergy



$R = 0.878$, $R^2 = 0.772$, $p = 0.0008$

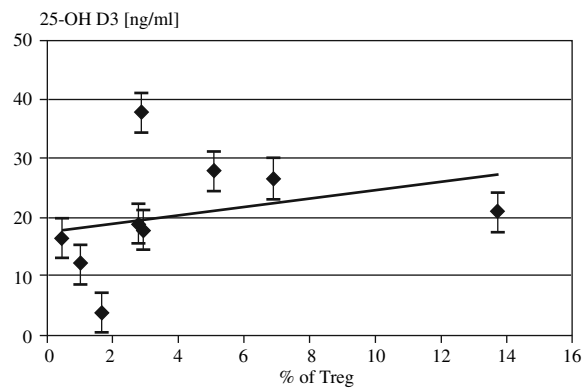
Fig. 3. Correlation between the percentage of regulatory cells (as percent of CD4 cells) and 25-OHD_3 in children with allergy symptoms

level of $p = 0.0008$. A similar dependence was not revealed in the group of children without allergy symptoms.

Discussion

Hypersensitivity and allergy are diseases associated with malfunction of the immune system. Chronic allergic inflammation, in the past called allergy, is excessive and changed response of the immune system, that definition was introduced by K. von Pirquet in 1906 [18]. Currently it is used as a synonym for temporary or permanent disturbances of immunoregulation causing incorrectly addressed immune response against a variety of allergens, leading to tissue damage. These diseases are called "civilization diseases" and affect increasing numbers of occupants, including children [19, 20]. Disturbing is the fact that the age of children catching severe forms of allergic diseases is decreasing. Recent studies have shown that there is subpopulation of T cells called regulatory cells (Tregs) responsible for the strength and scope regulation of the immune system response. Numerous studies have demonstrated that an increased Tregs subpopulation percentage reduces the sensitivity of the immune system to external or changed in the disease process antigens, which allows the growth of tumors [21, 22]. On the other hand decreased Tregs percentage increases the strength of immune response, which may lead to chronic inflammatory diseases and autoimmune diseases [23-25]. Therefore it seems to be important to find factors which can affect the level and function of the cells Tregs.

In the project, preliminary study was attempted to examine the relationship between serum 25-OH vitamin D_3 level and the value of Tregs in the blood cells of children with clinical symptoms of allergy. The control group was children hospitalized at the Clinic due to diseases with obturation, but without symptoms of allergies. The clinical diagnosis was a criterion for group division. Those children whose IgE levels were more than 2.5 times higher than the upper limit of normal for the particular age group were qualified to allergic group. This level is usually correlated with atopic diseases where IgE antibodies play a crucial role in response to the sensitizing antigen [26-29].



$R = 0.290$, $R^2 = 0.084$, $p = 0.449$

Fig. 4. Correlation between the percentage of regulatory cells (as percent of CD4 cells) and 25-OHD_3 in children with no symptoms of allergy (control group)

In performed experiments no statistically significant differences in both average number of leukocytes in the blood, and their respective populations (lymphocytes/monocytes/neutrophil/eosinophil/basophils) were observed. Also we didn't noticed significant differences in the mean concentration of 25-OHD_3 and the average number and percent-

age of regulatory cells in the blood between groups. Lymphocyte phenotyping revealed only statistically significant decrease of CD4 percentage in allergy group. Interesting information was provided by examination of correlation between serum 25-OHD_3 and the percentage of Tregs in the blood. There was no correlation in the control group (children without allergy), while in the group of children with allergies a strong, proportional correlation between serum 25-OHD_3 and the percentage of Tregs in the blood have been shown. Similar results in the studies of the correlation of these factors in multiple sclerosis were obtained [30]. There are also studies that do not support these observation. The research of Royal group's showed an inverse correlation with 25-OHD and directly proportional relation of Tregs and 25-$(OH)_2$D/25-OHD ratio [31]. Therefore, there is no conclusive data demonstrating the relationships between vitamin D metabolites and size of the sub-population of regulatory cells in the case of multiple sclerosis. It seems that these discrepancies may be the result of the lack of a clear methodology for determining of regulatory cell subpopulations.

Many other studies suggest an inverse relationship between low vitamin D levels in patients and increased incidence of various diseases, such as diseases of the skeletal system, cardiovascular, infectious diseases and autoimmune diseases [32-36]. Despite the high interest in the role of Tregs and vitamin D there is a lack of research on their significance in allergic diseases.

In the literature there is a lot of information about vitamin D deficiency and its negative effects (airway hyperresponsiveness, impaired respiratory function and resistance to steroid therapy), that may result from [37]. It is known that T cells play a key regulatory role in T-cell homeostasis and promote a correct immune response [38, 39]. It is widely believed that immunological basis of chronic allergic inflammatory according to inappropriate responses of T-cell helper 2 (Th2) to common allergens. Cytokines such as IL-4 and IL-13 associated with Th2 are responsible for IgE production in response to allergens, and IL-5 (also associated with Th2) contributes to eosinophilic inflammation, which is one of the characteristic features of allergic diseases [40, 41].

Given the above information and the results of the study (our own and others authors) it can be presumed that there is a potential for preventing, or at least reducing the symptoms of allergic diseases through proper supplementation of vitamin D.

There is still some open issues. Is the severity of allergy symptoms in the early spring season is only associated with an increased risk for allergens exposure, or is it also results from the low levels of vitamin D_3 (small sun exposure during the winter)? Does it matter that our study was performed between January and April? Why the correlation occurs in the group of allergic children only and was not observed in the control group (children without symptoms of allergy)? Are there any other immunological factors (changes in intracellular and surface markers, signal transduction disturbances, etc.), which may be useful in the diagnosis and treatment of atopic diseases? All these questions require further, much more detailed researches.

The results cannot be basis for the final conclusions (mainly due to small number of participants in both groups) but allow to have a hope for promising results in the future. The above data might suggest that Tregs cells are involved in T cell responses to allergic inflammation, and can play a possible role as a limiting element of this process. Also very important (especially in children) is the possibility to find an easy, cheap and effective method for reducing the allergic symptoms throught the adequate vitamin D_3 supplementation.

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