Early and late activation markers on thymus--dependent lymphocytes and natural killer cells in the blood of children with adenoid hypertrophy and concomitant otitis media with effusion

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Abstract

Adenoid hypertrophy is one of the reasons of otitis media with effusion. The aim of this study was to inquire whether it has got an influence on the number and activity of peripheral blood T and NK cells. The examined group consisted of 119 children with adenoid hypertrophy. The first group consisted of 44 children with only one episode of otitis media with effusion (OME). The second group consisted of 45 children with more than 4 episodes of OME and the third group consisted of 30 children with adenoid hypertrophy without OME. Evaluation of percentage of CD4+, CD8+ and NK subsets with co-expression of CD69, HLA-DR molecules in peripheral blood was performed by flow cytometry method. In examined groups with OME the decrease of CD4 number was compensated by increase of the number of NK and CD19 cells. In these groups the increase of CD69 and HLA-DR expression on the examined subsets was observed. The results suggest that recurrent otitis media with effusion may be one of the factors causing T lymphocytes deficiency. Obtained results might be one of the criteria of using adenoidectomy.

Key words: *otitis media with effusion, lymphocytes subsets, peripheral blood, flow cytometry.*

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Introduction

The immune system provides protection against infection and regulates processes of destruction and regeneration of body tissues; it plays the role of "the inner doctor". Our health depends on the efficiency of this "doctor", which is the sum of efficient mechanisms of defense and regeneration; it enables proper function of individual organs and body systems.

Properly functioning system does not allow an assault against its own normal tissues (auto aggression) or against foreign neutral agents (allergy), it directs its assault against pathogenic infectious agents. It also has a positive influence on its own tissues, helping in the regeneration process. Destructive mechanisms are defensive instruments and constructive mechanisms are regenerative instruments of the immune system. The right address is necessary for these mechanisms to function appropriately.

The efficiency of the immune system is essentially conditioned by the degree of immune competence. This we define as the ability to recognize and properly respond to

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potentiation or inhibition of immunologic reaction depending on the identified needs.

Respiratory tract infections account for about 50% of illnesses in children up to 5 years of age and about 30% in children between 5 to 12 years of age. The highest average number of respiratory tract infections (7 to 9) is observed between 2 and 4 years of age, whereas in the age group 8-10 years average is about 4 to 6 recurrences of infection during the year [1]. One of the causes of recurrent infections, especially recurrent otitis media with effusion (OME) is adenoid hypertrophy. Pharyngeal adenoid hypertrophy in recurrent OME may be the cause and the result of chronic inflammation process within the middle ear. About recurrence of OME, to a large extent, decide the specifics of the mechanisms of infection, interaction between cells of the mucosa and the virulence of microorganisms. On the other hand, the intensity, the extent and duration of the infection is decided by the host immune response. In case of frequent infections, where antibiotics do not prevent recurrences one should consider the possibility of impaired immune mechanisms, such as immunoglobulin deficiency, impaired function of phagocytes or quantitive and functional disturbance of thymus dependent lymphocytes [2-4].

In view of this data, in this study we attempted to answer the question of how pharyngeal adenoid hypertrophy, and associated otitis media, are reflected in percentage rate and the expression of early and late activation antigens on subsets of peripheral blood lymphocytes. This evaluation may be the basis for expanding the indications for a possible adenoidectomy.

Material and methods

Patients

The survey involved 119 children of both sexes, aged from 2 to 6 years with pharyngeal adenoid hypertrophy. First group included 44 children, which had an incident of OME, in no more than 3 months preceding the study. Second group included 45 children who had at least 4 episodes of OME during 6 to 12 months preceding the study. Third group included 30 children, who had pharyngeal adenoid hypertrophy and OME was not observed. Patients were examined during remission of illness. University Bioethics Commission gave its consent to the study.

Morphological evaluation of peripheral blood

For the evaluation of the differential white blood cell count 2 ml of venous blood was taken to a test tube with EDTA. Differential white blood cell count analysis was performed in the hematological analyzer (MAXMEM, Coulter, Germany). Leukocytosis was given in the G/L, the element quantity of differential white blood cell count is given in percentage ratio (%) and absolute (BL, in the G/L) values.

Evaluation of peripheral blood lymphocyte subpopulations. With the rest of the samples of morphological venous blood a cytometric analysis was made. To 100 µl of whole blood 10 µl of following monoclonal antibodies were added (Dako): CD3-RPE-Cy5/CD4-FITC/CD8-RPE, CD4-RPE-Cy5/CD69-FITC/HLA-DR-RPE, CD8-RPE-Cy5/CD69-FITC/HLA-DR-RPE, CD16+56-RPE-Cy5/CD69-FITC/HLA-DR-RPE. For each test a set of isotype negative controls was used. After 15 min of incubation at room temperature, each sample had undergone a rapid, automatic lyses (ImmunoPrep Work Station, Coulter). After thorough mixing, the samples were analyzed by flow cytometry method (Coulter Epics XL), counting 10^4 cells each time. The results were given as percentage ratio and based on the data from differential white blood cell count – absolute values.

Statistical analysis

Statistical analysis was calculated according to ANOVA/MANOVA procedures. The differences between the arithmetic means of each age group, for each of the evaluated parameters were analyzed by Mann-Whitney nonparametric ranking test, accepting $p < 0.05$ as significant difference.

Results

In the first stage, an evaluation of the number of leukocytes, percentage ratio and absolute value of peripheral blood lymphocytes was done. The results of the evaluation are presented in Table 1.

In the absence of significant differences between the statistical calculations performed for average percentage and absolute number of lymphocytes, in subsequent stages of the test, statistical analysis was done on the percentage ratio of leukocyte subpopulation only. In the next stage of the test, percentage ratio of peripheral blood lymphocyte subpopulations was assessed. The results of the evaluation are presented in Table 2. In the case of CD19, CD3, CD4 and CD8 positive cells no significant differences were observed between groups 1 (i.e. children with pharyngeal adenoid hypertrophy, who had 1 incident of acute otitis media) and children in group 3, who had pharyngeal adenoid hypertrophy without incidents of otitis media. A statistically significant difference from group 1 was observed in group 2, i.e. children with pharyngeal adenoid hypertrophy, who during the period from 6 months to 2 years had at least 4 incidents of acute otitis media with effusion. Within this group there was a significantly higher percentage ratio of CD19 cells, NK cells, a lower ratio of TH/TS cells and the lower percentage of CD3 and CD4 cells. There were no significant differences in regards to the percentage of CD8 cells.

The percentage ratio of CD4, CD8 and NK cells with co-expression of receptor CD69 (early) and HLA-DR (late) activation was subsequently assessed. In evaluating the sub-

Table 1. Leukocytes and lymphocytes in children peripheral blood

Parameters	Group 1: otitis media with effusion	Group 2: reccurent otitis media	Group 3: adenoid hypertrophy	Statistical analysis
Number of leukocytes (G/L)	8.9 ±2.6	9.7 ±1.2	8.1 ±2.3	NS
Percentage of lymphocytes (%)	31.1 ±12.5	27.1 ±12.9	35.7 ±14.1	NS
Number of lymphocytes (G/L)	2.75 ±0.8	2.3 ±0.76	2.76 ±0.8	NS

Data are presented as mean ±SD

Table 2. Lymphocytes subsets in children peripheral blood

Group	Percentage of lymphocytes subsets					
	CD19	CD3	CD4	CD8	NK	TH/TS
1	15.4 ±3.1	73.1 ±3.8	40.7 ±4.1	26.9 ±4.7	7.4 ±3.87	1.4 ±0.1
2	24.6 ±57.1	63.1 ±8.1	32.1 ±3.6	31.3 ±8.2	10.1 ±4.8	1.05 ±0.2
3	19.7 ±4.2	62.8 ±4.8	36.2 ±5.0	30.9 ±8.0	6.4 ±3.5	1.25 ±0.39
Statistical analysis	1 vs. 2 $p < 0.01$	1 vs. 2 $p < 0.02$	1 vs. 2 $p < 0.01$	NS	2 vs. 3 $p < 0.009$ 1 vs. 3 NS	1 vs. 2 $p < 0.05$

Data are presented as mean ±SD. Group 1 – otitis media with effusion, group 2 – recurrent otitis media, group 3 – adenoid hypertrophy

Table 3. Lymphocytes subsets with co-expression of CD69 and HLA-DR in children peripheral blood

Group	Percentage of lymphocytes subsets					
	CD4-HLA	CD8-HLA	NK-HLA	CD4/CD69	CD8/CD69	NK/CD6±
1	4.69 ±1.6	11.3 ±6.4	9.7 ±5.2	3.4 ±1.8	3.8 ±2.3	9.7±3.2
2	6.39 ±2.2	21.3 ±15.7	22.2 ±9.4	4.2 ±2.7	5.2 ±1.4	14.4 ±9.5
3	3.1 ±1.3	10.15 ±4.3	3.7 ±1.8	1.6 ±0.8	2.7 V1.5	4.8 ±2.2
Statistical analysis	NS	1 vs. 2 $p < 0.003$ 2 vs. 3 $p < 0.005$	1 vs. 2 $p < 0.006$ 2 vs. 3 $p < 0.002$	1 vs. 3 $p < 0.01$ 2 vs. 3 $p < 0.01$	1 vs. 3 $p < 0.05$ 2 vs. 3 $p < 0.001$	2 vs. 3 $p < 0.01$ 2 vs. 3 $p < 0.01$

Data are presented as mean ±SD. Group 1 – otitis media with effusion, group 2 – reccurent otitis media, group 3 – adenoid hypertrophy

population of lymphocytes with HLA-DR co expression receptor, statistically significant differences were found in CD8 and NK lymphocyte subsets, where a group of children with pharyngeal adenoid hypertrophy and recurrent OME had significantly higher average values in relation to two other groups. The percentage leukocyte subpopulation with co-expression of receptor CD69 has the highest average values in both groups; particularly, significantly higher in group 2 for NK, CD4 and CD8 lymphocytes.

Discussion

The aim of these study was to evaluate the level of various lymphocyte subpopulations and the presence of early and late activation markers on T lymphocytes and NK cells in children with pharyngeal adenoid hypertrophy accompanied by OME.

The purpose of this evaluation were lymphocytes from peripheral blood of children with pharyngeal adenoid hypertrophy with incidental OME (group 1) and frequent OME (group 2). Obtained results were compared to a group of children with pharyngeal adenoid hypertrophy without OME (group 3). To optimalize conditions adopted in this study patients with OME were evaluated over a period of remission.

In the first stage of this test we characterized population of peripheral blood lymphocytes. The studies assessed

the percentage ratio of – CD3+, CD4+, CD8+ – T cells; percentage ratio of – CD19+ – B cells and CD3-CD16+56+-NK cells. Activation of lymphocytes was evaluated by assessing surface expression of HLA-DR and CD69 antigens.

In the group of children with OME, at the same time there was a decrease in percentage of CD3+ cells and an increase in the percentage of CD19+ cells and NK cells. Based on the results, one can not determine whether the observed quantitative shortage of CD3+ cells is the cause or effect of the observed clinical symptomatology? In the case-by-case study, the factor that suppresses the immune system, particularly its component cells, appears to be chronic antigenic stimulation. On the other hand, the cause of this suppression could be antibiotics taken during the infection, which are highly ineffective, given the recurring nature of the process and deepens an existing immune deficiency [5-9].

Based on previous studies [10, 11] quantitative reduction and functional impairment of T lymphocyte populations by the use of most antibiotics, it can be assumed that adverse effects of antibiotics correspond to the term "stage thymectomy" [12].

The dominance of the process of inflammation in the group of children with OME states a significantly higher proportion of CD8+ cells and NK cell with co-expression receptor HLA-DR – late activation antigen. The increase of HLA-DR expression in the assessed cases is probably caused by chronic antigenic stimulation. It is difficult, on the basis of these tests to judge, the increase in the percentage of NK cells and NK cell with HLA-DR co-expression receptor, is by how much compensatory to T-cell deficiency, and to how this compensation meets the needs of the immune system in conditions of forced stimulation during recurrent OME infection in children. The question remains open of the quality of compensatory responses of NK cells, in their participation in an immune response to which they are biologically characterized and predisposed. Attempting to answer this question, one should assess the expression of early activation antigen – CD69, for the evaluated lymphocyte subsets.

In the studies of percentage ratios of lymphocytes with antigen CD69 co-expression, in a group of children with pharyngeal adenoid hypertrophy and recurrent OME in all lymphocyte subsets i.e. CD4+ cell, CD8+ and NK cells, significantly higher values were obtained in relation to both other groups.

It has been proven that the CD69 antigens manifest *in vitro* under the influence of a wide range of stimuli in most hemopoetic cell lines, *in vivo* CD69 antigen expression is mainly observed in inflammatory sites mainly in T lymphocytes [13]. In the phenotypes of dormant CD3+ lymphocytes manifestation of CD69 antigen in healthy subjects ranges from 5% to 12% [14]. In the cytoplasm of dormant T lymphocyte, CD69 antigen occurs in preformed stages, and thus its surface expression, in the initial phase of activation does not require mRNA synthesis de novo [13, 14].

In *in vitro* studies, a correlation was observed between the dose of antigen Alloiococcus otitidis and the degree of expression of CD69 antigen in T lymphocytes from peripheral blood. The highest antigenic stimulation of CD69 molecule was observed in CD8+ lymphocytes [15, 16].

Also, in the studies evaluating the lymphocytes from pharyngeal tonsil in patients with OME, an increase in expression of early and late activation antigens, which would correspond to chronic antigenic stimulation was reported [17-19]. Similar studies evaluating the subpopulations of leukocytes isolated from effusion fluid from the middle ear have shown a correlation between the increase in the inflammatory process in pharyngeal tonsil and local inflammatory process within the middle ear [20-23].

In the present study we observed that recurring OME with pharyngeal adenoid hypertrophy is accompanied by varying degree of immune deficiency, mainly in a lymphocytes T population. This deficit is compensated by the rise in NK cells and CD19 cells. Based on these studies, it is difficult to conclude the primary or secondary character of observed changes. These changes, however, seem to be a one more indication for surgery in cases where pharmacotherapy does not provide the expected results.

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