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RSV RESPIRATORY INFECTION IN CHILDREN UNDER 5 Y.O. - DYNAMICS OF THE IMMUNE RESPONSE Th1/Th2 AND IgE

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ABSTRACT

INTRODUCTION. The imbalance of Th1/Th2 cytokine concentrations and increased level of IgE might be useful tool for prediction of severity of RSV infection among young children and possibility of sequels. The ratio of cytokines Th1/ Th2 varied during the disease.

THE AIM of our studies was the assessment of immunological response by dynamics of Th1 and Th2 cytokines and IgE in RSV infections.

MATERIAL AND METHODS. Study was done on sera collected from 36 young children hospitalized because of RSV infection and from 16 children with other respiratory tract infection (HMPV, EV, HPIV1-3). Assaying of the serum levels of cytokine Th1 (IL-2, IFN-g, TNF), Th2 (IL-4, IL-6, IL-10) and concentration of IgE has been done. Paired sera (48 patients) were collected in the interval 4-14 days. Reference group consist of 18 children (<6 months of life) hospitalised because other than respiratory diseases with negative results for viruses tested by PCR.

RESULTS. Among children with respiratory infection the Th1/Th2 ratio was shifted towards Th2, level of IgE increased in comparison to the reference group. Changes in concentration of IL-6, IFN-g, IL-10 were related to RSV infection, mainly bronchitis and bronchiolitis, while the dynamic of TNF concentration was independent on aetiological agent. It was found that the risk factors (prematurity, artificial nutrition) correlated with RSV bronchitis and the levels of cytokines and IgE. Increased level of IL-6 and IL-10 were shown in prematures, and increased concentration of IgE – among artificial nourished children. The time of serum collection affected the level of cytokines and IgE and the effect was depended on the aetiological agent. In RSV infections was observed significant decrease with time of IL-6, IL-10 and IFN- γ but not IgE (still significantly higher than in the reference group). While the significant decrease of IgE was determined only in other than RSV infections.

CONCLUSIONS. Determined level of cytokines and IgE varied depending on the time of serum collection. Observed dynamics in paired sera and IgE might have prognostic value in disease and sequels of RSV infections. Prevention RSV infection in premature infants should be done in any possible way. Breastfeeding is one of the critical elements of prevention of RSV infection.

Key words: *Th1/Th2 balance, dynamics of cytokines and IgE, RSV infection, RNA viruses in respiratory infection, artificial feeding*

INTRODUCTION

RSV infection of infants, young children under the age of 2 years, and the elderly may have severe course threatening the patient's life. They may also cause other remote complications (1,2). Studies done by other researches indicated the increased level of Th2 cytokines and IgE in the serum of patients infected with RSV. It suggests that activation of mechanisms, similar to those induced by allergens, occurs during the RSV infection.

It was supposed that soluble viral glycoprotein G (SG) plays role of super-antigen for T cells. This antigen is capable of binding to a domain VH3IgE on the hematopoietic cells, basophils, mast cells and mononuclear cells. Like the allergens this aggregation causes the release of large amounts of Th2 cytokines into the blood thereby balance of Th1/Th2 is removed towards Th2 > Th1. A characteristic feature of Th1 lymphocyte is interferon gamma (IFN- γ), IL-2 and TNF production. Interleukins produced by Th2 lymphocytes include IL-4,

IL-5, IL-6, IL-9, IL-10 and IL-13. The balance between Th1/Th2 is also regulated by cytokines: IFN- γ stimulates Th1 response by inhibition of the function and proliferation of Th2 cells. Simultaneously IL-4 secreted by Th2 lymphocytes stimulates these cells together with IL-10 and IL-13 by inhibition of cytokine production by Th1 cells (cellular response suppression) (3,4,5,6).

The purpose of this study was to analyze the relations between the levels of cytokines IL-2, IL-4, IL-6, IL-10, TNF, IFN-g, and IgE in the serum samples of two groups of children with respiratory tract infections. First group consisted of patients with infections caused by RSV, second - patients who have had an acute respiratory infection caused by RNA viruses - other than RSV. We evaluated the effect of elapsed time between onset of disease and material sampling on the levels of cytokines and IgE. We also analyzed the relations between the clinical symptoms and levels of cytokines and IgE in both groups of patients.

MATERIALS

Altogether 120 serum samples were collected: from 52 children, under 5 years old, hospitalized for an acute respiratory tract infection (tested group) and from 20 children hospitalized for other reasons (reference group).

Tested group: 52 children with acute viral respiratory infections (ARTI). Study group was divided into two groups: 36 children with RSV infection confirmed by RT-PCR and with dynamic of IgM antibodies specific for RSV (the first group); and 16 patients with infection caused by other than RSV RNA virus confirmed by RT-PCR and without IgM antibodies specific for RSV (second group). Nine patients had *enterovirus* (EV) infection, 4 children - parainfluenza type 1,2 or 3 (HPIV 1-3), and in 3 children - *human metapneumovirus* (HMPV) infections were detected.

Among examined children 60% were under 6 months of life, 30% were in the age from 6 to 12 months, and only 5 children (10%) were older. Girls constitute 58% of whole group. From 48 patients following data from the questionnaire were obtained: date of illness, date of collection of serum samples, diagnosis/ symptoms occurred during the infection (pneumonia, bronchiolitis, bronchitis, sore throat, wheezing, fever, runny nose, shortness of breath, cough, dry cough, symptoms of gastrointestinal system, symptoms of the nervous system) and risk factors of RSV infection (prematurity, low birth weight).

The first serum samples (52 children) were collected at 1-10 days from the date of onset of respiratory symptoms. The second one- at 4-14 days from the date of the first serum sampling, was collected from 48 children

for whom the full survey data have been obtained. The second serum samples were tested to determine the influence of the time between onset and of sample collection on the level of cytokines and IgE.

Reference group: Reference group included 20 children less than 6 months of age, without symptoms of RTI, hospitalized for the other reasons than respiratory infection (mostly hyperbilirubinemia). From all of the children the nasopharyngeal swabs were collected and tested for the presence of RSV, HMPV, EV, HPIV1-3 by RT-PCR method. Two children were excluded from further analysis because of the positive RT-PCR results for RSV.

METHODS

Determination of cytokine level. Determination of IL-2, IL-4, IL-6, IL-10, TNF, IFN-g in all sera from patients was performed by a flow cytometry method in the Becton Dickinson FACScalibur flow cytometer using BD™ Cytometric Bead Array (CBA) Human Th1/Th2 cytokine kit II (Becton-Dickinson, NJ, USA). Overall, the test was performed in 120 serum samples which were stored at minus 70°C and have not been earlier thawed. The assays were performed according to the manufacturer's instructions.

Determination of IgE levels. IgE levels were determined by ELISA IgE (NT-DNOV Immunodiagnostica-Nova Tec GmbH) according to the manufacturer's instructions.

Analysis of the questionnaire data (diagnosis, symptoms, risk factors), identified infectious agents and the obtained laboratory results was performed using Statgraphics for Windows, Centurion, v.XV. StatPointTech. Inc.USA. Multiple variables analysis method were used for quantitative data. For data without normal probability distribution the comparison of medians-Mann-Whitney (Wilcoxon) W test and Kolmogorov-Smirnov Test were used. The multiple variable comparison included ANOVA, Multiple Range Tests (based on 95% LSD), Kruskal-Wallis Test and Mood's Median Test were done. For qualitative/categorical data crosstabulation, tests of independence (χ^2 or Fisher's exact tests), the degree of association between rows and columns (Contingency Coeff., Lambda test and Pearson's correlation), When appropriate odds ratios and relative risk were calculated. Relation between categorical variables were analyzed using Poisson correlation method. The relation was considered as significant at $P_0 < 0.05$.

RESULTS

Symptoms/ diagnosis. The analysis of data included in questionnaire (diagnosis and symptoms) allowed for

relatively precise characteristics of the study group with regard to clinical symptoms. In 68% of the children pneumonia was finally diagnosed, bronchiolitis in 14.5%, bronchitis in 78%, in 56.5% - pharyngitis. The most common symptoms were: runny nose (74% children), productive cough (71%), dyspnea (68.5%), wheezing (49%), fever > 38°C (43%), dry cough (22%). Fever, shortness of breath, productive cough were not observed among children from the reference group (significant, $P_0 < 0.05$). Gastrointestinal symptoms were observed in 14.5% of children from the studied group. There were found associations between sex and the occurrence of: 1. bronchitis ($P_0 = 0.0013$, more frequently in boys); 2. fever (more common in girls $P_0 = 0.0307$); 3. dyspnea (more frequently in boys $P_0 = 0.0413$) and 4. symptoms from the gastrointestinal tract ($P_0 = 0.0359$). Moreover, the occurrence of fever was depended on the age of patients ($r = 0.2731$, $P_0 = 0.0232$) and was higher in older children. The mutual analysis of recognition/symptoms revealed that in patients with pneumonia there were no bronchiolitis ($P_0 = 0.0369$), cold ($P_0 = 0.0279$), gastrointestinal symptoms ($P_0 = 0.0396$), whereas dyspnea ($P_0 = 0.0011$) was observed. In patients with bronchiolitis - fever ($P_0 = 0.0000$) and runny nose ($P_0 = 0.0428$) were more common, but a lower incidence of pharyngitis ($P_0 = 0.0113$) was found. The bronchitis and wheezing ($P_0 = 0.0013$) were frequently observed together.

Cytokine levels. In the reference group - the analysis of inter-relations between cytokine levels indicated significant correlation between IL-2 and IL-6 ($r = 0.9754$, $P_0 = 0.0000$), IL-2 and TNF ($r = 0.9987$, $P_0 = 0.0000$), IL-2 and IFN-g ($r = 0.8793$, $P_0 = 0.0000$), IL-6 and TNF ($r = 0.9737$, $P_0 = 0.0000$), IL-6, and IFN-g ($r = 0.8803$, $P_0 = 0.0000$) and TNF and IFN-g ($r = 0.8769$, $P_0 = 0.0000$). There were no correlations between IgE levels and the level of any of the determined cytokines.

Test results of determination of cytokine concentration in the study group showed a very high diversity. The level of cytokines/IgE and the data from the questionnaire, the identified infectious agents and time of sampling were analyzed with chosen statistical tests.

Analysis of cytokines and IgE levels and diagnosis/symptoms and risk factors. Analysis of the results depending on the etiological agent of infection revealed that among children infected with RSV the level of IgE was higher than in the reference group and patients infected with viruses other than RSV ($P_0 = 0.036$). Simultaneously, it was found that the higher levels of IgE occurred in older children infected with RSV ($r^2 = 0.30$, $P_0 = 0.023$). RSV infection has been associated not only with increased levels of IgE ($P_0 = 0.0005$), but with higher level of IL-10 ($P_0 = 0.0001$) and IL-6 ($P_0 = 0.030$). There were no significant correlations between the level of IL-2, IL-4, IL-5, TNF, IFN- γ and the detection of RSV as the agent of respiratory infection. In RSV infection

– the significantly higher level of IL-10 was observed not only in relation to the reference group but also to the group of patients with infection caused by other RNA viruses. Such relation for TNF was not observed. The relations between risk factors of patients and the level of IgE or cytokines were also analyzed. Higher level of IgE was found in artificially fed infants ($P_0 = 0.03$) and in children with bronchopulmonary dysplasia ($P_0 = 0.006$). In these children significant correlation between Th2 response (expressed as the sum of the related results) and IgE level ($r^2 = 0.70$, $P_0 = 0.002$) were found, whilst this relation was not observed in other children.

The dynamics of the level of cytokines and IgE depending on the time of sample collection. Determined level of cytokines in the first serum sample (1-10 days after onset) and the second serum samples (4-14 days from the date of the first sera collection) were compared. Concentration of cytokines and IgE in serum of children obtained in the study group showed high diversity therefore the results are presented as a ratio of average values of the median in reference group (median of multiple determination of each variable in the reference group) (tab.I). In the first serum samples the level of IL-2 and TNF was below median of the reference group, and the level of other cytokines and IgE was higher than in the reference group. The differences in the levels of cytokines and IgE were significant, although for the IFN- γ and TNF demonstrated by only one of the many used statistical methods (tab.I). The highest differences in cytokines levels in the first serum samples compared to the reference group were observed for IL-6 and IL-10. In the second sera these differences were lower than in the first sera, but still significant.

In the second serum samples lower cytokines levels than in the reference group were found only for IL-2 ($P_0 < 0.05$). Statistically significant increased level of TNF was observed in the second samples in comparison to both: the reference group ($P_0 < 0.05$), and the result obtained in the first serum samples ($P_0 < 0.05$). Statistically significant decrease of the level of IL-6 was demonstrated in the second sera in comparison to the level observed in the first serum samples, although simultaneously the increase of IL-6 compared to the reference group was still significant. No differences in the level of IFN- γ , IL-4 and IgE depending on the time of samples collection were found.

Analysis of the relations between the level of cytokines in the first serum samples and clinical symptoms (multivariate methods) showed ($r^2 = 43.7\%$, $P_0 = 0.0000$) association between bronchitis and the level of IL-10 ($P_0 = 0.0106$), IFN-g ($P_0 = 0.0292$), and IgE ($P_0 = 0.0021$). In the second sera only a relation between bronchitis and IgE level ($P_0 = 0.006$) was found.

The effect of serum sampling time on the concentration of interleukin and IgE was also analyzed (without division into groups: the first or second samples). The

Table I. Determined cytokine and IgE levels in paired serum samples from 48 children in the study group. Analysis of the significance of differences for the median value of the corresponding assay in the reference group (median in the reference group = 1)

The results of the determination of cytokines and IgE levels	Cytokines Th1			Cytokines Th2			IgE
	IL2	IFN- γ	TNF	IL4	IL6	IL10	
First serum sample							
The ratio of average / median reference +/- Standard deviation	0,26 +/-1,30	5,6 +/-13,5	0,32 +/-0,96	8,6 +/- 34,6	74,6 +/-165,5	17 +/-24,0	11,3 +/-26,9
Significance of differences in relation to reference group	Z	Z*	Z*	Z	Z	Z	Z
Second serum sample							
The ratio of average / median reference +/- Standard deviation	0,16 +/-0,82	1,32 +/-4,13	17,4 +/-117,3	1,96 +/-8,6	7,1 +/-37,5	7,05 +/-22,9	6,39 +/-14,66
Significance of differences in relation to reference group	Z	N	Z	N	Z	Z	Z*
Significance of differences in relation to results of first serum sample	N	N	Z	N	Z	Z*	N

Abbreviations:

Z – statistically significant differences ($P_0 < 0.05$); significantly higher values; significantly lower values; N – no statistically significant differences ($P_0 > 0.05$); Z* - significant differences only in one of the methods of differentiations

Table II. Relations between time of serum sampling (the number of days from the date of onset) and determined cytokines and IgE levels in children infected with RSV and other viruses RNA (nRSV)

Examined factor	Virus	Function of time dependent	r	P_0
IL-6	RSV	$Y = a + b \cdot \sqrt{X}$	0,4420	0,0008
	nRSV	no	ns	0,1981
IL-4	RSV	no	ns	0,2478
	nRSV	no	ns	0,7220
TNF	RSV	no	ns	0,3370
	nRSV	no	ns	0,8485
IFN	RSV	$Y = (a + b \cdot X^2)^2$	0,2853	0,0365
	nRSV	no	ns	0,5436
IL-10	RSV	$Y = (a + b \cdot X)^2$	0,4130	0,0019
	nRSV	no	ns	0,1814
IgE	RSV	no	ns	0,6885
	nRSV	$Y = (a + b \cdot X^2)^2$	0,5813	0,0002

results of the analysis of the function of IgE /interleukin concentration dynamics of (Y) depending on the time interval (X) between the onset and samples collection were presented in table II.

The dynamics of the IL-6, which is a significant decrease in the level of IL-6 over time from the date of onset of symptoms, was observed in RSV infection, regardless of the diagnosis/clinical symptoms. A similar relations were observed for IFN- γ and IL-10. However, a significant decrease of IgE level was observed as a function of time only in children with infections caused by virus other than RSV. Determined IgE levels was still at a high level and higher than in the reference group in the group of children with RSV infection.

DISCUSSION

The clinical symptoms of RSV infections are the result of many different factors such as the proper-

ties of the virus, environmental factors (eg. smoking in patient's community), patient's risk factors (eg. prematurity) and genetic polymorphisms of patients (1,7). Genetic predispositions of the patient are often a key factor determining the immune response to RSV infection (8), including the Th1/Th2 balance. Bronchial hyperreactivity and asthma are caused by shift of Th1/Th2 balance towards Th2 and therefore may be an example of prior RSV infection in the past (9). In patients with RSV infection, in whom the balance moved toward Th2 responses, the subsequent occurrence of complications is very likely. Some authors point out the role of allergin (IgE) and its prognostic role in predicting the late consequences of RSV infections (3). There were published a lot of articles presenting the results of study on the immune response to RSV infection. The most important difficulties in the analysis of these information are both: clinical diversity of tested materials such as sera (9), bronchoalveolar lavage-vesicular (10,11), as well as profile of examined interleukins: IL-2 (9), IL-4 (12), IL-5 (12), IL-6 (13), IL-8 (14,11), IL-9 (15), IL-10 (15), IL-13 (12), IFN-(16), TNF (15). Moreover, the time component was found as an important factor in the experiments of animal models (17). This is a result of both: the dynamics of infection and increase of cytokines and IgE as well as their interactions (4). The analysis of literature indicated that the impact of the time of sampling was omitted in most of the studies of young children.

One of the purposes of this study was to compare Th1 and Th2 responses and the level of IgE in respiratory tract infections in young children caused by RS virus and other RNA viruses. The levels of cytokine IL-2, IL-4, IL-6, IL-10, TNF and IFN were determined as representative of both processes as allergies and leading to asthma (4). In addition, we analyzed the influence of time of sample collection on the concentration of the

cytokines (the time variable) based on examination of paired sera. The time between sample collection and onset of symptoms of respiratory infection was taken under consideration. In our study, the general characteristics of Th1 (IL-2, IFN, TNF) and Th2 (IL-4, IL-6, IL-10) response in the respiratory tract infections caused by RSV virus was similar to those described by other authors (5,3), who reported the shift of balance towards Th2 responses. This shift was found both in relation to the reference group and the respiratory diseases caused by RNA viruses other than RSV. The growth of TNF level in later phase of disease, above 10 days after the onset of symptoms, regardless of the etiologic agent was the interesting observation.

Similarly to other researches, in sera of patients with viral respiratory tract infections, particularly patients with bronchitis, higher level of IgE than in the reference group was observed (3). Besides RSV infection, in the case of other viruses respiratory tract infections, the level of IgE showed a statistically significant decrease over time of the onset of symptoms. In RSV infections the stable level of IgE of both serum samples were observed.

We analyzed also the relations between the immune response in infections caused by RSV or other RNA viruses, and with clinical diagnosis, symptoms of respiratory infection, and the collected data for any factors which may have influence on the course of the disease. Multivariate analysis of such data indicated the significant correlations between IgE levels ($r^2 = 76\%$, $P_0 = 0.0000$), and bronchitis ($P_0 = 0.006$) as well as bronchiolitis ($P_0 = 0.012$) in RSV infections. This relation has not been observed in patients with pneumonia ($P_0 = 0.214$). Furthermore, analysis of the relations between the level of cytokines in serum (in the first samples) and clinical symptoms ($r^2 = 43.7\%$, $P_0 = 0.0000$) showed the relations between the level of IL-10 ($P_0 = 0.0106$) and IFN ($P_0 = 0.0292$), and diagnosed bronchitis.

In the analysis of the risk factors of RSV infection in children it was found the significant correlation between the RSV infection and the circumstances preceding the infection ie bronchopulmonary dysplasia ($P_0 = 0.037$) and artificial nutrition ($P_0 = 0.017$). Among preterm infants with bronchitis caused by RSV determined levels of IL-6 ($P_0 = 0.0077$), and IL-10 ($P_0 = 0.0024$) were significantly higher. A similar correlation was observed in patients with pulmonary bronchopulmonary dysplasia ($P_0 = 0.0020$). We also found that higher levels of IgE in respiratory infection may be associated with low birth weight ($P_0 = 0.035$) and the artificial feeding ($P_0 = 0.023$). These observations may support the hypothesis of *Juntti* et al, that the susceptibility to RSV infection as well as the type of induced by infection response are the results of child's prior immune status (9). Simulta-

neously, it indicates that the key role in preventing the RSV infections is breast-feeding of preterm infants.

CONCLUSIONS

1. Risk of contact / infection by RSV in the first months of life premature infants should be minimized. It is important to maintain breast-feeding of premature infants wherever it is possible.
2. More attention should be paid to the influence of time between infection onset and serum sampling for determination of the level of cytokines and IgE.
3. It was shown that the examination of cytokine concentration in paired patient's sera taken at an interval of 6-10 days may have prognostic importance for the course of infection due to RSV and late complications of this infection.

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