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Efficacy of combined immunotherapy with propes and inflamafertin in selective deficiency of NK and NKT cells in children with autism spectrum disorders associated with genetic deficiency of the folate cycle.

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Abstract

The purpose of the research was to study effectiveness of combined immunotherapy with Propes and Inflamafertin in NK and NKT cell deficiency in children with autism spectrum disorders associated with genetic deficiency of the folate cycle. This single-center, prospective, controlled, nonrandomized clinical trial included 96 children aged 2 to 10 years with autism spectrum disorders associated with a genetic folate deficiency (study group, SG). Children of SG received Propes at a dose of 2 ml IM every other day for 3 consecutive months (45 injections), and Inflamafertin at a dose of 2 ml IM every other day for 3 months in a row, alternating with Propes (45 injections). The number of NK cells reached the lower limit of normal in 39 out of 53 patients (74% of cases), with the resulting deficiency of these lymphocytes, and the average number of NK cells in the blood in SG almost doubling during the 3-month course of immunotherapy ($p<0.05$; $Z<Z_{0.05}$). The number of NKT cells was normalized in 78 out of 87 patients (89% of cases) with an initial deficiency of these cells, and the average number of NKT cells in the blood in the DG increased during the course of immunotherapy by half ($p<0.05$; $Z<Z_{0.05}$) and continued to grow for the next 2 months after the discontinuation of immunotropic drugs ($p<0.05$; $Z<Z_{0.05}$). Combination immunotherapy with Propes and Inflamafertin is effective strategy for the treatment of immunodeficiency caused by genetic deficiency of the folate cycle in children with autism spectrum disorders.

Keywords: Autoimmune complications, Cell immunodeficiency, Combined immunotherapy, Immunomodulation, Immunoprophylaxis.

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Introduction

Data from recent meta-analysis of randomized controlled clinical trials indicate an association between autism spectrum disorders and genetic folate deficiency in children [1,2]. It is established that the genetic deficiency of the folate cycle affects the immune status of children with autism spectrum disorders, forming a kind of immunodeficiency, which is based on a decrease in the number and functional activity of Natural Killers (NK cells) and Natural Killer T Cells (NKT cells) [3]. Immunosuppression due to genetic deficiency of the folate cycle mediates the development of a number of immune-dependent complications that determine the formation of inflammatory encephalopathy in children with autism spectrum disorders, in particular–reactivated opportunistic infections [4,5]. Autoimmune reactions against neurons and myelin [6,7] and systemic inflammation with cytokine release syndrome with hypercytokinemia [8,9]. The compensation of immunodeficiency, induced by genetic deficiency in the folate cycle, appears to be an attractive prospect for preventing or at least alleviating the manifestations of associated immune-related complications that affect the severity of CNS damage in children with autism. However, such therapeutic approaches remain undeveloped and are therefore not available to patients. The results of previous small clinical trials indicate the potential benefit of combined immunotherapy with Propes and Inflamafertin to compensate for the deficiency of NK- and NKT-cells in folate cycle deficiency [10,11]. However, this encouraging data should be validated in larger controlled clinical trials with greater weight of the results obtained. Propes–a biological agent containing alpha-and beta-defensins, has a pronounced immunoactivating and lymphoproliferative effect. At the same time, Inflamafertin, which contains alarmines and adrenomedulin, in contrast has an anti-inflammatory effect that is mediated by interleukin 10, which is important in preventing autoimmune complications in drug-induced immune activation. Based on the experience of usage of another highly active immunomodulatory agent-recombinant interleukin 2–therapeutic immune activation can cause an undesirable increase in the risk of autoimmune complications [12]. Therefore, the combination of immunoactivating drug Propes with an anti-inflammatory tolerogenic immunotropic agent seems to be the key to achieving safe immunomodulatory therapeutic effect.

Purpose

To study the effectiveness of combination immunotherapy with Propes and Inflamafertin in NK and NKT cell deficiency in children with autism spectrum disorders associated with genetic deficiency of the folate cycle.

Materials and Methods

This single-center, prospective, controlled, nonrandomized clinical trial included 96 children aged 2 to 10 years with autism spectrum disorders associated with genetic folate deficiency. These patients formed the Study Group (SG). The diagnosis of autism spectrum disorders was made by psychiatrists of regional hospitals or specialized departments according to the criteria of DSM-IV-TR (Diagnostic and Statistical Manual of mental disorders) and ICD-10 (The International Statistical Classification of Diseases and Related Health Problems). The basis for including a patient in this trial was the written consent of the parents for the child's participation in the study (Protocol No 128 dated 23.12.2019 from the Commission on Bioethics of the Bogomolets NMU).

To verify the genetic deficiency of the folate cycle, the following nucleotide substitutions in the folic acid cycle genes were determined: MTHFR 677 C>T, MTHFR 1298 A>C, MTRR 66 A>G i MTR 2756 A>G in various combinations in the homo- and heterozygous state by PCR with restriction. In such children, persistent hyperhomocysteinemia was recognized-a serum concentration of homocysteine above the level of 5.2 μmol/l, which is a biomarker of folate cycle deficiency. The number of NK and NKT cells in the blood was measured by laser flow cytofluorimetry (cytometer Epics Xl, USA), using the method of indirect immunofluorescence and monoclonal antibodies to CD markers of lymphocytes (triple label; reagents Beckman Coulter, USA). NK cells meant a subpopulation of lymphocytes with a phenotype CD3-CD16+CD56+, and with NKT cells - a subpopulation of lymphocytes with a phenotype CD3+CD16+CD56+. At the beginning of the study, NKT-cell deficiency occurred among patients in the study group in 84% of cases, while NK-cell deficiency-in 51% of cases, including combined deficiency of NKT-and NK-cells-in 35% of cases (Figure 1). Immune status studies were performed monthly for 5 consecutive months during a 3-month course of immunotherapy, and for additional 2 months after the completion of immunotherapeutic interventions.

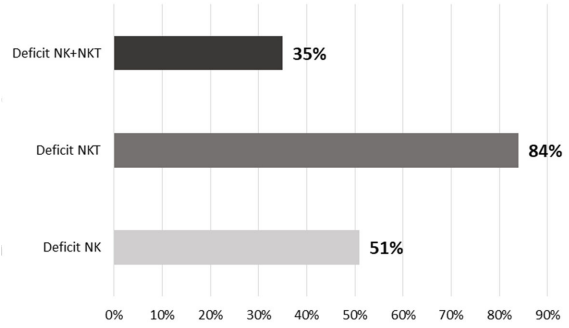

Figure 1. The structure of SG (n=96) by deficiencies of NKT- and NK-cells in the blood.

The children of the SG, due to NK- and/or NKT-cell deficiency, received the tested combination immunotherapy. Propes was administered at a dose of 2 ml IM, every other day for 3 consecutive months (45 injections). Accordingly, Inflamafertin was administered at a dose of 2 ml IM, every other day for 3 consecutive months, alternating with Propes (45 injections).

The control group (CG) consisted of 32 children of similar age and gender distribution who suffered from autism spectrum disorders associated with genetic deficiency of the folate cycle, but did not receive immunotherapeutic interventions to compensate for the deficiency of NK and NKT cells. These children received only the currently recommended educational development programs in specialized centers for patients with special needs.

Methods of structural and comparative analyzes were used for statistical analysis of the obtained information. In order to establish the reliability of differences in results, the Student's T-test was used to calculate the confidence probability of coefficient p (parametric criterion) and the number of Urbach signs Z (nonparametric criterion). To study the relationship between the assignment of immunotherapy and the dynamics of the studied indicators of immune status, the Pearson Chi-square (χ^2) was calculated with the determination of the Yates correction. To determine the strength of the detected bonds, the criterion φ (F-test), the Pearson correlation Coefficient (C) and its normalized value (C') were also calculated. To verify the obtained data, the calculation of the Odds Ratio (OR) and 95% confidence interval (95% CI) were used. The information was processed using Microsoft Excel.

Results and Discussion

The results of structural analysis in the observation groups indicate that the number of NK cells reached the lower limit of norm in 39 out of 53 patients (74% of cases), with an initial deficiency of these lymphocytes. And the average number of NK cells in the blood in SG almost doubled over the 3 months course of immunotherapy, but returned almost to baseline levels in the 2 months after the discontinuation of immunotherapeutic agents. In contrast, the number of NKT cells was normalized in 78 of 87 patients (89% of cases) with an initial deficiency of these cells. Also, the average number of NKT cells in the blood in the SG increased during the course of immunotherapy by at least half and continued to grow steadily for the next 2 months after discontinuation of the tested immunotropic drugs, almost doubling at the end of the observation period.

Data from comparative and variational analyzes indicate a probable difference in the average number of NK cells in the blood in SG and CG during the period of 1-3 months of immunotherapy ($p<0.05$; $Z<Z_{0.05}$), but not after the discontinuation of immunotherapeutic drugs. A significant difference in the mean numbers of NKT cells in the blood in the observation groups occurred between 2 and 5 months of the study ($p<0.05$; $Z<Z_{0.05}$), but not after the first months of treatment, continuing for at least 2 months following the discontinuation of the tested immunotropic agents (Figures 2 and 3).

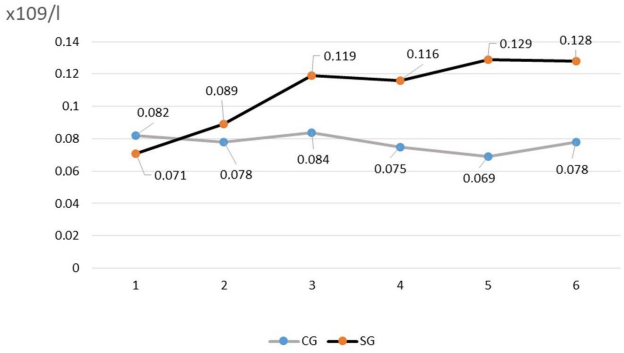

Figure 2. Dynamics of the number of NKT cells in the blood in observation groups during the clinical trial.

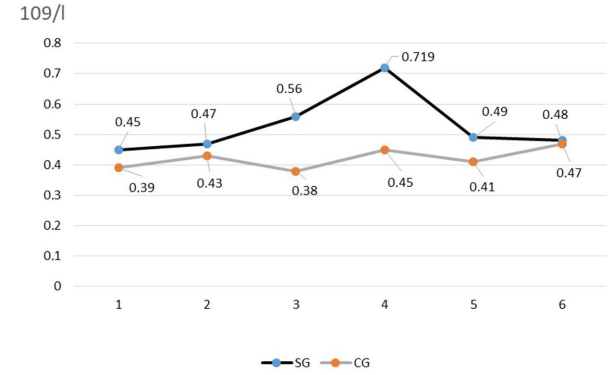

Figure 3. Dynamics of the number of NK cells in the blood in observation groups during the clinical trial.

Conclusion

Literature on joint attention shows that monitoring children's cognitive abilities in early stages is of fundamental in order to early detect autism predictors. However, the present study shows that certain temperamental patterns, which are manifested since birth, have a potential impact on the development of JA showing that monitoring predictors of autism can be done even earlier than JA skills manifestation.

Therefore, we believe that temperamental patterns need to be included in the toolkit for early detecting autism manifestations. Moreover, we hope that this toolkit might be implemented also by parents, and not only by practitioners, if considering the easiness and low-cost of administration. Although it is premature to draw conclusions with respect of the possibility that temperamental patterns contribute to a very early detection of autism spectrum disorder, we hope that the present study might be of special help to caregivers into improving the environmental factors that affect temperament, and consequently, the development of JA.

Compliance with Ethical Standards

All procedures performed in this study (which involved human participants) were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent Statement

Informed consent was obtained from all caregivers of the participants included in the study. The data obtained indicate the ability of combination immunotherapy to increase the number of NK and NKT cells in the blood of children with autism spectrum disorders that are associated with genetic deficiency of the folate cycle, normalizing their immune status. However, the patterns of response from different subpopulations of lymphocytes to the tested immunotherapeutic agents differ from each other's immune status. Thus, the NK cells respond to immunotherapy faster and more intensely, but the effect is short-lived and lasts only against the backdrop of applied immunotherapy, while the number of NKT cells in the blood grows more slowly with a 1-month delay. However, a prolonged positive effect is achieved, because the gradual increase in the number of these lymphocytes in the blood persists even during the first 2 months after the discontinuation of immunotropic drugs.

To test the association between combination immunotherapy and normalization of the number of NK and NKT cells in the blood, we studied Pearson's chi-square (χ^2), the Yates-corrected chi-square test, and the plausibility-adjusted chi-square test. This data would allow to determine whether the applied immunotherapeutic interventions were the cause of the resulting changes in the immune status of SG patients. Based on the data, the number of NKT cells was restored in 78 out of 87 SG patients with initial deficiency of these lymphocytes, and only in 5 out of 32 CG patients with deficiency of these cells before the start of the study. And the number of NK cells reached the norm in 39 out of 53 patients with corresponding deficiency in SG, and only 3 out of 18 people with the initial deficiency in CG. Obtained results are presented in Table 1.

Indicator	NK-cells		NKT-cells	
	Value	Probability	Value	Probability
chi Pearson square	18.016	<0.001	60.65	<0.001
chi-square with Yates correction	15.737	<0.001	57.307	<0.001
chi-square adjusted for plausibility	18.613	<0.001	60.282	<0.001

Table 1. Evaluation of chi (χ^2) Pearson's square and other indicators of contingency between immunotherapy and normalization of immune status in SG patients (n=96).

The obtained results (Table 1) indicate a link between immunotherapy and the achievement of normalization of impaired immune status-the number of NK- and NKT-cells in the blood - in children with autism spectrum disorders associated with genetic deficiency of the folate cycle. This indicates that the prescribed immunotropic drugs were the most likely cause of positive changes in the immune status of SG

patients. To study the strength of relationship between the tested immunotherapeutic interventions and the normalization of studied indicators of immune system, the values of the coefficient φ, the Pearson correlation coefficient and its normalized value were calculated. This would allow us to assess the efficiency of Propes and Inflamafertin on the immune system in children with autism spectrum disorders associated with genetic deficiency of the folate cycle. The obtained results are contained in Table 2. As can be seen in Table 2, there was a predominantly strong or relatively strong association between immunotherapy and the changes in achieved immune status, indicating a high efficacy of the tested immunotherapeutic agents in the SG. NKT cells were found to be slightly more sensitive to combination immunotherapy than NK lymphocytes, although both subpopulations of lymphocytes showed convincing evidence of a strong association between immunotherapy and normalization of blood counts.

To verify the data on the strong association between immunotherapy and normalization of NK and NKT cell counts in SG patients, an odds ratio (OR), a standard error of the odds ratio (S), and a 95% confidence interval (95% CI) were calculated. This would avoid errors in assessing the contingency between the studied processes in previous stages of statistical analysis. The obtained results are presented in Table 3.

Indicator	NK-cells	NKT- cells		
	Degree of Association	Indicator	Degree of Association	
F^*-criterion	0.504	relatively strong	0.715	strong
Pearson's correlation coefficient (C)	0.45	relatively strong	0.581	relatively strong
Normalized value of the Pearson correlation coefficient (C')	0.636	strong	0.822	very strong

Table 2: Evaluation of F-test and other indicators of degree of association between immunotherapy and normalization of immune status in SG patients (n=96).

Indicator	NK-cells	NKT-cells
Odds ratio (OR)	13.929	46.8
Standard error of the odds ratio (S)	0.705	0.601
95% confidence interval (95% CI)	3.498-55.468	14.415-151.937

Table 3. Evaluation of Odds Ratio (OR) and other indicators of the relationship between immunotherapy and normalization of immune status indicators in SG patients (n=96).

As can be seen from Table 3, the calculation of OR and 95% CI confirms the previously obtained results regarding the close relationship between the tested immunotherapy and normalization of studied indicators of immune status in SG patients. It is a once again demonstrated fact, already identified in the previous stage of statistical data analysis, regarding higher sensitivity of NKT cells compared to NK lymphocytes in the combined immunotherapy of Propes and Inflamafertin in the SG.

This data indicates an appropriate immunomodulatory effect of the tested immunotherapeutic strategy in specific form of immunodeficiency, which is observed in children with autism spectrum disorders associated with genetic deficiency of the folate cycle. Immunodeficiency in children with autism spectrum disorders is most likely responsible for the development of a number of immune-related complications that affect both the severity of mental disorders and the overall health. In particular, it is an abnormally high microbial load on the body [4,5] persistent immunoinflammatory enterocolitis [13] a tendency to generate allergic manifestations [14] systemic inflammation with hypercytokinemia [8,9] and autoimmune reaction against neurons and myelin [6,7]. Normalization of impaired immune status is the key to preventing the development of a number of immune-dependent complications in children with autism spectrum disorders, which will improve their clinical condition and can significantly improve the response to neuroprotective therapeutic strategies [15-17].

Conclusion

The results obtained in this controlled non-randomized clinical trial indicate that combination immunotherapy with Propes and Inflamafertin is an effective treatment strategy for immunodeficiency caused by genetic folate deficiency in children with autism spectrum disorders. These biological immunotropic drugs are able to normalize the previously reduced number of NK and NKT cells in the blood in this category of patients during a 3-month course of immunotherapy, with a more frequent, stronger and more lasting effect on NKT cells compared to NK lymphocytes.

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