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12 EFFICACY OF RITUXIMAB IN AUTISM SPECTRUM DISORDERS ASSOCIATED WITH GENETIC DEFICIENCY OF THE FOLATE CYCLE WITH SIGNS OF ANTINEURONAL AUTOIMMUNITY

JUSTIFICATION

Advances in genetics, molecular biology, and immunology over the past decades have significantly changed our understanding of the etiology and pathogenesis of autism spectrum disorders (ASD) in children. One of the key advances in this direction is the elucidation of the association of genetic deficiency of the folate cycle (GDFC) with ASD, evidence for which is based on the results of at least 5 meta-analyses of randomized controlled clinical trials and a number of additional controlled trials, the data of which have not yet been properly summarized [14, 21, 27, 28, 29]. It has been established that GDFC leads to pathological biochemical changes in the child's body, which determine the development of encephalopathy with the clinical picture of ASD due to direct (metabolic) and indirect (immune-mediated) mechanisms, and immune-dependent pathways of cerebral damage are currently given a leading role in the pathogenesis of this mental disorder. Among the metabolic disorders induced by GDFC in the child's body, hyperhomocysteinemia, vitamin deficiencies, signs of mitochondrial dysfunction, and impaired nucleotide synthesis and DNA, protein, and lipid methylation processes are distinguished [12, 31, 32]. These pathological biochemical changes lead to the development of persistent oxidative stress, as evidenced by the results of two systematic reviews and meta-analyses of randomized controlled clinical trials on this problem [6, 9]. The result of such disorders are the phenomena of neuro- and immunotoxicity, which underlie the above-mentioned direct and indirect mechanisms of neuronal damage in children with ASD. If we talk about immunotoxicity, it is currently established that in GDFC there is a disturbed development of the child's immune system with the formation of immune dysfunction and dysregulation, which, in turn, cause a phenomenon called a disturbed neuroimmune interface [19, 25]. It is believed that there are at least three main immune-mediated mechanisms of brain damage in GDFC, which can radically affect the development of associated encephalopathy with ASD symptoms. Neurotropic opportunistic and conditionally pathogenic infections [23], autoimmune reactions to neurons, myelin, glial cells of the cerebral hemispheres and cerebral vessels [5, 10], systemic and associated intracerebral persistent aseptic inflammation mediated by existing immune dysregulation [18, 30], constitute the indicated triad of key pathogenetic mechanisms of the development of ASD-forming encephalopathy in GDFC. Suppression or even eradication of these immune-dependent GDFC-induced pathways of CNS damage currently appears to be a promising prospect for effective treatment of ASD in children with GDFC. In particular, it is believed that the suppression of autoimmunity and neurons and myelin can significantly improve the mental functions of sick children. A number of clinical studies have already been conducted in this direction. In particular, clinical case reports and the results of small trials have shown the benefit of using glucocorticosteroids and some other anti-inflammatory agents in children with ASD, the mechanism of action of which is seen precisely in the implementation of anti-inflammatory action and suppression of anti-brain autoimmunity [17]. At least 10 clinical studies have been conducted to test the immunomodulatory agent intravenous normal human immunoglobulin in ASD, which is believed to improve mental functions

of patients by suppressing intracerebral inflammation and autoimmune reactions against brain autoantigens [1, 3, 4, 7, 8, 12, 13, 16, 20, 24, 26]. Recently, infliximab, a monoclonal antibody against the tumor necrosis factor alpha molecule, has demonstrated efficacy in suppressing hyperactivity and hyperexcitability in children with ASD associated with GDFC in a controlled clinical trial [2].

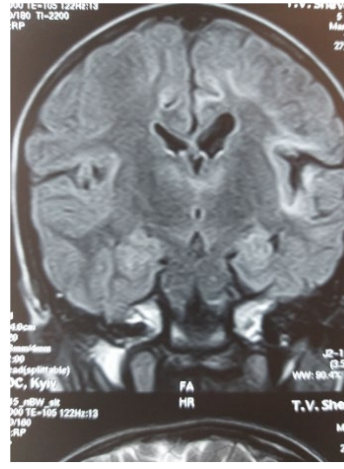
The prospect of developing new, more effective and safe methods of treating immune-mediated encephalopathy in children with ASD is an important task of modern neuroimmunology. Given that autoimmune reactions to CNS autoantigens in ASD are believed to be mainly mediated by autoantibodies rather than cellular autoimmune reactions, the monoclonal antibody to the CD20 molecule of B lymphocytes, rituximab, which has already undergone a number of successful trials in autoimmune diseases with a similar mechanism of development, seems promising for use in such children. Theoretically, by inducing B-cell depletion, rituximab can significantly suppress or even eliminate the production of autoantibodies to brain autoantigens in children with ASD, having a neuroprotective effect and thereby improving the mental status of patients. A dedicated clinical trial testing rituximab in children with ASD associated with GDFC and evidence of anti-brain humoral autoimmunity is needed.

The aim of the research: to study the effectiveness of rituximab in children with ASD associated with GDFC, who have serological signs of antineuronal autoimmunity, to expand the current arsenal of neuroprotective therapy for immune-mediated encephalopathy in such cases.

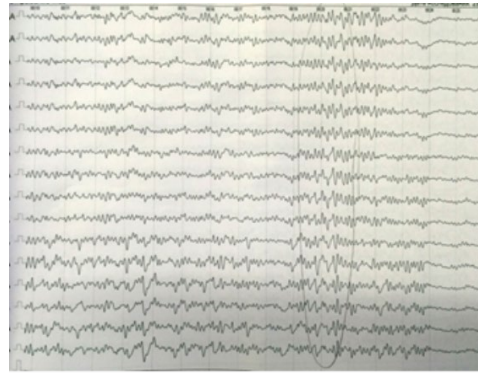
Materials and methods. The medical data of 225 children aged 3 to 9 years with GDFC, who had clinical manifestations of ASD, were analyzed. All of them were patients of the specialized neuroimmunological clinic Vivere (registration dossier dated 12/22/2018 No. 10/2212–M). Data for the study and processing of the material were carried out in accordance with contract No. 150221 dated 02/15/2021, and the conclusion of the bioethical examination commission (protocol No. 140 dated 12/21/2020, Bogomolets NMU). The diagnosis of autism spectrum disorders was made by child psychiatrists 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). Pathogenic polymorphic variants of folate cycle enzyme genes were determined by restriction PCR based on the detection of the MTHFR C677T nucleotide substitution in monoform (27 patients), as well as – in combination with other nucleotide substitutions – MTHFR A1298C, MTRR A66G and/or MTR A2756G.

The results of serological studies of blood serum were evaluated for the detection of specific antineuronal autoantibodies, which are validated as markers of autoimmune limbic encephalitis in children and adults, namely autoantibodies to glutamic acid decarboxylase (GADA), neuronal potassium channels, amphiphysin, NMDA–receptors of neurons, GABA, CV2, Yo, Ro, Hu, AMPAR 1 and 2. Positive results of such laboratory studies were found in 81 patients.

These results were combined with signs of hyperintensity of the MR signal from the structures of the mesolimbic system of the temporal lobes of the cerebral hemispheres in the T2 and FLAIR modes during MR neuroimaging (**Fig. 12.1**), as well as with the EEG pattern of temporal median epilepsy during neurofunctional studies (**Fig. 12.2**). Since these individuals had signs of autoimmune limbic encephalitis, with which the existing clinical neuropsychiatric disorders could be associated, they were offered treatment with rituximab according to the latest systematic review on the problem of therapy of the indicated autoimmune lesions of the CNS [22].



⬡ **Fig. 12.1.** MRI image of bilateral autoimmune limbic encephalitis with asymmetric hippocampal and insular involvement associated with the production of autoantibodies to neuronal potassium channels in a child with ASD associated with GDFC (FLAIR mode, coronal projection; own observation)



⬡ **Fig. 12.2.** EEG pattern of epileptiform bioelectrical activity associated with GADA autoantibody-induced temporal median sclerosis in a child with ASD associated with GDFC (pathological waves are circled; own observation)

The parents of 62 of 81 ASD patients with signs of antineuronal autoimmunity agreed to the proposed immunotherapy. Their children formed the study group (SG). Autoantibodies to GADA were present in 30 patients (48 %) of SG patients, to potassium channels of neurons in 24 people (39 % of cases).

Autoantibodies to amphiphysin (3 people, 5 %), NMDA receptors of neurons (3 people, 5 %) and CV2 molecules (2 people, 3 % of cases) were also rarely found (**Fig. 12.3**). Relatives of the other 19 patients with a similar distribution of antineuronal autoantibodies refused such treatment (control group, CG).

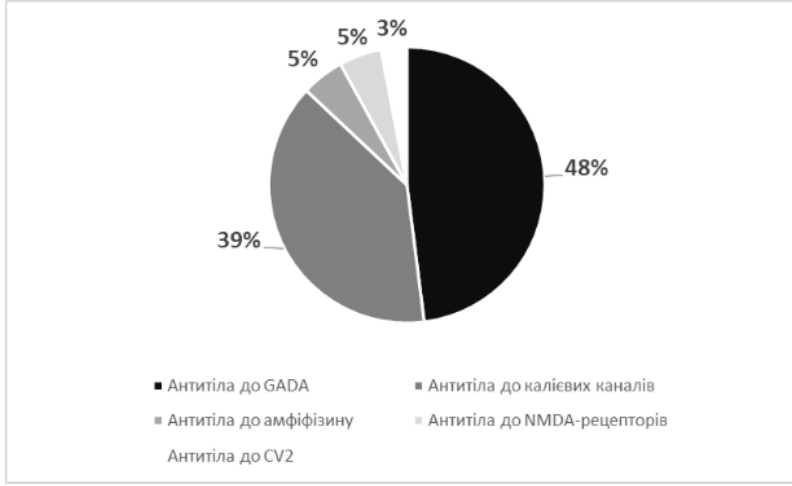


Fig. 12.3. Structure of SG (n = 62) by type of serum autoantibodies to CNS neuron autoantigens

Since serum concentrations of various antineuronal autoantibodies were measured in different units, a special scoring system was used to conduct a generalized data analysis. Exceeding the serum concentration of a particular autoantibody by up to 20 % of the upper limit of reference values was evaluated as 1 point, from 21 to 40 % – 2 points, from 41 to 60 % – 3 points, from 61 to 80 % – 4 points, and more than 81 % – 5 points. Since autoantibodies to GADA and neuronal potassium channels were found in many patients, a separate analysis of the data was performed for these indicators, which could not be carried out for autoantibodies to neuronal NMDA receptors, amphiphysin and CV2 due to the small number of cases of their identification among the examined patients.

Rituximab, a monoclonal antibody preparation to the CD20 molecule of B lymphocytes, was administered intravenously drip at a dose of 375 mg/m^2 of the child's body surface with a frequency of 1 time per 1 month under the control of the results of determining the serum concentrations of autoantibodies to autoantigens of neurons of the mesolimbic system of the brain until the disappearance of such autoantibodies from the child's blood serum. In total, from 3 to 9 courses of rituximab immunotherapy were performed in SG children.

The dynamics of clinical symptoms of ASD were assessed according to the specialized Aberrant Behavior Checklist (ABS) scale in SG and CG children in order to determine how much the decrease in serum concentrations of antineuronal autoantibodies affects the clinical status of patients.

Statistical processing of the material was carried out by comparative and structural analyses. To determine the probability of differences between the studied indicators in the observation groups, the parametric Student's T-test with the confidence probability indicator p and the non-parametric criterion – the number of signs Z according to Urbach Yu.V. were used. To study the association of the dynamics of serum concentrations of antineuronal autoantibodies and indicators of cerebral damage in children with ASD, the odds ratio (OR) and 95 % confidence interval (95 % CI) were calculated.

Microsoft Excel was used for statistical calculations.

The study was carried out as a fragment of research work commissioned by the Ministry of Health of Ukraine (state registration number 0121U107940).

Research results and their discussion. Data from the structural analysis of the results of the use of approved rituximab immunotherapy among patients of the observation groups with signs of antineuronal autoimmunity indicate that the normalization of previously elevated serum concentrations of antineuronal autoantibodies in SG children after a 3-month course of rituximab immunotherapy was noted in 37 % of cases, after a 6-month course – in 79 % of cases, and after a 9-month course – in 92 % of cases, while in CG similar indicators corresponded to the levels of 7, 11 and 14 % of cases, which was a significant difference from SG ($p < 0,05$; $Z < Z_{0,05}$) (**Fig. 12.4**). The average course of rituximab immunotherapy in SG was 4.91±0.65 months.

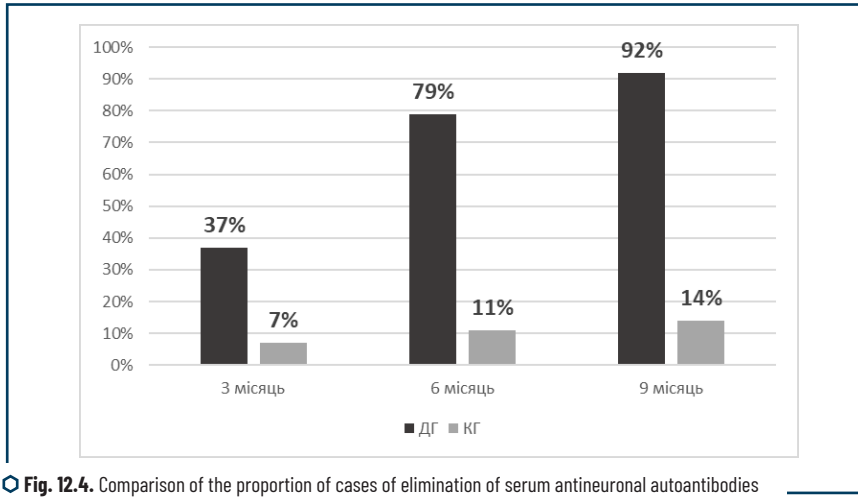


Fig. 12.4. Comparison of the proportion of cases of elimination of serum antineuronal autoantibodies in patients SG (n = 62) and CG2 (n = 19) during the course of rituximab immunotherapy

Although there was a small proportion of spontaneous normalization of serum concentrations of antineuronal autoantibodies in CG, immunotherapy with rituximab was associated with a 5-fold increase in the number of cases of obtaining normal serum concentrations of these autoantibodies after 3 months of immunotherapy, an 11-fold increase after 6 months of immunotherapy, and more than 14-fold increase after 9 months of use of the approved monoclonal antibody preparation.

Thus, the use of rituximab was associated with a progressive increase in the cases of negating previously positive results of serum antineuronal autoantibody measurements in SG children as the course of immunotherapy continued. Only 8 % of SG children showed resistance to a 9-month course of rituximab. Additional analysis showed that all of these children had a maximum antineuronal autoimmune response score (5 points) at the time of starting immunotherapy, indicating a high intensity of the autoimmune response. All of these children had decreased serum antineuronal antibody concentrations by the 9th month of immunotherapy by at least 60 %, indicating partial, rather than total, resistance to the immunotherapeutic interventions.

The results of the study of the monthly dynamics of the score of the intensity of the antineuronal autoimmune reaction indicate that throughout the entire course of rituximab, a progressive decrease in the serum concentration of antineuronal autoantibodies was noted in SG children. Thus, the average score of the assessment of the antineuronal autoimmune reaction in SG2 before the start of immunotherapy was 4.32±0.27 points, while after a 9-month course of therapy it was only 1.31±0.14 points, which indicated a decrease in the intensity of the total autoimmune reaction by almost 4 times, although in CG there was no significant dynamics of the score of autoimmunity against CNS neurons (4.01±0.26 and 4.46±0.47 points, respectively), which indicated a significant difference between the results of the observation groups ($p < 0,05$; $Z < Z_{0,05}$) (**Fig. 12.5**).

There was a delay in the serological response to rituximab immunotherapy for at least 2 months from the start of immunotherapy, which can be explained by the period of complete decay of pre-existing autoantibodies to neurons synthesized by B lymphocytes before the start of immunotherapeutic interventions, which is about 42–46 days.

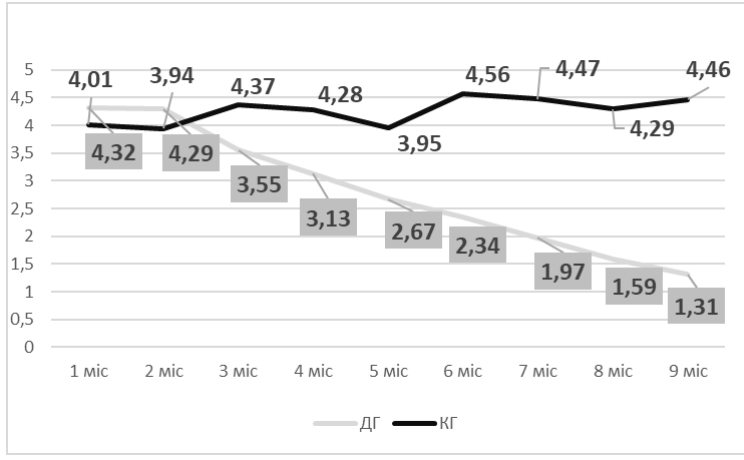


Fig. 12.5. Dynamics of serum concentrations of antineuronal autoantibodies in patients SG (n = 62) and CG (n = 19) during the course of rituximab immunotherapy

These data indicate that rituximab does indeed affect the severity of the autoimmune reaction against CNS neurons in children with ASD associated with GDFC. Moreover, the positive effect of immunotherapy develops rapidly, already during the first 3 months of immunotherapy, consistently increases as the course of immunotherapy continues and leads to the elimination of signs of autoimmunity in almost all cases.

The speed of achieving the endpoint – elimination of antineuronal autoantibodies from the patients' serum – depends on the initial level of their serum concentration, since 89 % of SG patients, in whom the disappearance of serological signs of autoimmunity was noted after the first 3 months of immunotherapy, had a low initial score of the autoimmune reaction at only 1–2 points, while all patients with partial resistance to rituximab, who underwent all 9 months of approved immunotherapeutic interventions without complete elimination of antineuronal autoantibodies from the blood serum, had a high initial score of 5 points ($p < 0,05$; $Z < Z_{0,05}$).

Additionally, a comparative analysis of the effectiveness of rituximab in antineuronal autoimmunity caused by autoantibodies to neuronal potassium channels and GADA was conducted, since this allowed for a significant number of similar cases in SG (**Fig. 12.6**). Separate analysis of the dynamics of serum concentrations of other autoantibodies to CNS neurons noted in SG children was made impossible by the small number of relevant observations.

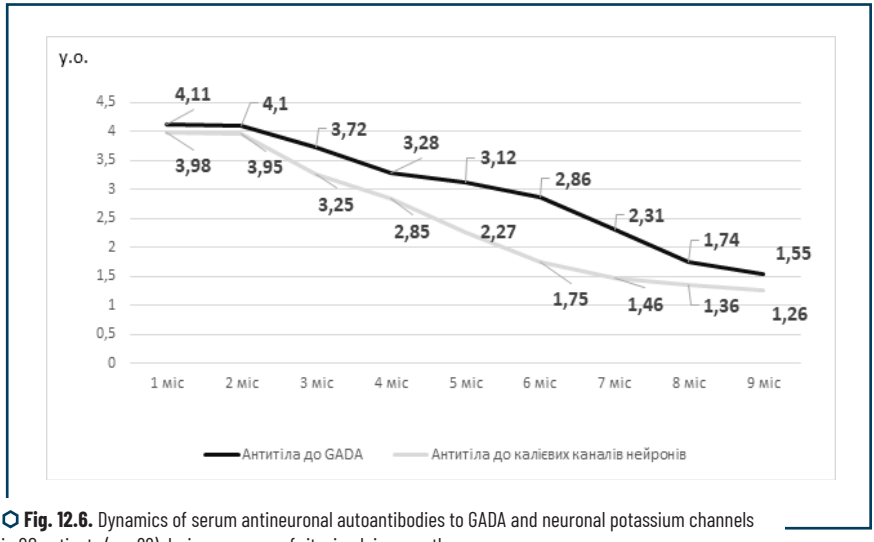


Fig. 12.6. Dynamics of serum antineuronal autoantibodies to GADA and neuronal potassium channels in SG patients (n = 62) during a course of rituximab immunotherapy

As shown in Fig. 6, rituximab was more effective in patients with autoantibodies to neuronal potassium channels than in patients with autoantibodies to GADA, although overall the efficacy of immunotherapy was quite high in both cases. These results are consistent with the established notion that rituximab is more effective in antineuronal autoimmunity caused by autoantibodies to surface autoantigens than by autoantibodies

to intracellular neuronal autoantigens, since in the latter case, cellular mechanisms of autoimmunity play a greater role in the pathogenesis of the disease, which are not affected by the monoclonal antibody drug used.

Therefore, when identifying serological signs of antineuronal autoimmunity to GADA in children with ASD associated with GDFC, a longer course of immunotherapy with rituximab should be expected than when detecting autoantibodies to neuronal potassium channels. Perhaps, to equalize the expected timing of immunotherapy in both cases, higher doses of rituximab should be initially used or standard immunotherapy should be combined with glucocorticosteroids specifically in patients with autoantibodies to GADA in the blood serum.

The fundamental question is whether the achieved phenomenon of rituximab-induced elimination of serum antineuronal autoantibodies is associated with the effect of neuroprotection. To this end, we studied the association of negativity of serological test results with normalization of previously elevated serum concentrations of cerebral damage biomarkers neuron-specific enolase (NSE) and S-100 protein (**Table 12.1**), the relevance of which has previously been demonstrated in specially designed controlled clinical trials in children with ASD [14, 32].

● **Table 12.1.** Results of the study of the association between the phenomenon of negativity of serological test results and normalization of serum concentrations of NSE and S-100 protein (OR; 95 % CI) in SG (n = 62)

Indicator	Antibodies to GADA	Antibodies to neuronal potassium channels
NSE	17,875; 4,738– 67,436	41,800; 7,257–240,778
S-100	9,750; 2,707–35,113	18,333; 3,462–97,083

As can be seen from the results of **Table 12.1**, the disappearance of serum autoantibodies to both GADA and neuronal potassium channels was associated with the normalization of previously elevated concentrations of both laboratory biomarkers of cerebral damage studied, which allows us to speak about the neuroprotective effect of immunotherapy with rituximab in children with SG. In the subgroup of patients with autoantibodies to neuronal potassium channels, a more pronounced association of the dynamics of the serological index and cerebral biomarker was noted compared to the subgroup of individuals with autoantibodies to GADA, which is consistent with the results of the analysis of the dynamics of serum concentrations of both types of antineuronal autoantibodies during the course of immunotherapy with rituximab in SG. At the same time, there was a closer association with NSE than the S-100 protein, which can be explained by the tropism of the detected anticerebral autoantibodies in children with SG. Since it was antineuronal autoantibodies that were noted, which primarily affect the gray matter of the brain, it was NSE, which characterizes neuronal damage, that turned out to be more informative, rather than the S-100 protein, the serum concentration of which increases with damage to the white matter of the cerebral hemispheres.

It was also important to investigate the clinical significance of the phenomenon of rituximab-induced disappearance of autoantibodies to CNS neurons in SG children, since there is still ongoing discussion about the role of antineuronal autoimmunity in the pathogenesis of ASD in children.

The data on the dynamics of the mental state score of children on the ABC scale indicate a significant improvement in all studied indicators in children receiving rituximab immunotherapy compared with CG patients. There was a decrease in the severity of clinical manifestations of hyperactivity and hyperexcitability, improvement in eye contact and behavior, progress in speech skills, and an increase in the overall score of the child's mental development. These clinical effects developed and deepened during the course of immunotherapy as serum concentrations of autoantibodies to CNS neurons decreased (**Table 12.2**).

● **Table 12.2.** ABC score in SG (n = 62) and CG (n = 19) patients after completion of rituximab immunotherapy

№	Subscales	SG (n = 62)	CG (n = 19)
	ABC		
1	Irritability	6.4±0.8*	14.1±1.5
2	Hyperactivity	10.9±1.4*	22.5±2.1
3	Inadequate eye contact	4.1±0.8*	8.6±1.3
4	Inappropriate speech	1.6±0.5*	7.9±1.5
	Symptom Checklist		
1	Drowsiness	5.7±0.7*	14.2±1.4
2	Decreased activity	1.7±0.4*	5.4±0.5

Note. * – $p < 0.05$: $Z < Z_{0.05}$

These data indicate that autoimmunity to CNS neurons is an important component of the pathogenesis of ASD in children with GDFC, and the elimination of serological manifestations of anti-neuronal autoimmunity with rituximab is associated with a significant improvement in the mental state of children. Therefore, immunotherapy with rituximab modifies the mental state of children with ASD associated with GDFC, uniformly affecting all major clinical signs of mental illness according to the ABC scale.

Conclusions. Rituximab treatment leads to a progressive decrease in serum concentrations of antineuronal autoantibodies in patients with GDFC-associated ASD, with a more pronounced effect in the case of autoantibodies to neuronal potassium channels compared to autoantibodies to GADA, with complete elimination of all types of autoantibodies from the serum of patients after a 9-month course of immunotherapy in at least 92 % of cases. The phenomenon of rituximab-induced elimination of serum antineuronal autoantibodies is associated with a neuroprotective effect, which is confirmed by the normalization of previously elevated concentrations of laboratory biomarkers of cerebral damage NSE and S-100 protein in serum. Most likely, it is the achieved neuroprotective effect that determines the progressive improvement in the main clinical manifestations of ASD in children with GDFC throughout the course of immunotherapy. The obtained data confirm the clinical significance of serum antineuronal autoantibodies in children with ASD associated with GDFC and indicate the effectiveness of rituximab for neuroprotection by suppressing anti-brain autoimmunity and achieving associated improvement in the mental status of the child in such cases.

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