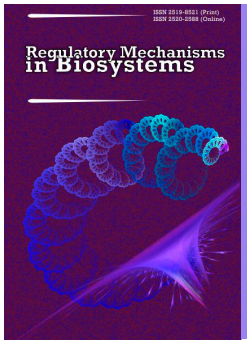

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Features of self-tolerance loss in patients with different clinical phenotypes of myasthenia

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The incidence of myasthenia gravis which is characterized by progressive muscular weakness on the background of structural disorders of the thymus, has increased. Myasthenia gravis is a multifactorial autoimmune disease, it has a pronounced clinical heterogeneity, and therefore the standard diagnostic and treatment protocol is not always effective. To substantiate an individual approach to the treatment of various clinical forms of myasthenia, we conducted a study of mechanisms and markers of loss of central and peripheral self-tolerance in thymus-independent myasthenia (M) and thymus-dependent myasthenia gravis with thymus hyperplasia (MH) and thymoma (MT), involving a total of 427 patients examined. In patients with different phenotypes of myasthenia, we used the methods of spectrophotometry, flow cytometry, enzyme immunoassay. In patients with MH on the background of lymphofollicular thymus hyperplasia we revealed a pronounced humoral sensitization in comparison with the reference values: the concentration of C_4 complement, C-reactive protein, circulating immune complexes and the initiation of an indirect autoimmune reaction – a reliable increase in autoantibodies (AAbs) to the α_1 and α_7 subunit of subunit of nicotinic receptors (nAChR). In M and MT groups a high similar titer of AAbs to other epitopes was revealed: DNA, β_2-glycoprotein I, membranes of intestinal and stomach cells, lung, liver, kidney cells. A pronounced blast-transforming response to the presence of the mitogen PHA was revealed in the MT group. In the MT group, a decrease in the content of CD^{4+} CD^{28+} co-stimulatory molecules and in the MH group, a decrease in CD^{4+} CD^{25+} Treg lymphocytes was revealed. Individual methods for correcting the loss of self-tolerance in patients with different clinical phenotypes of myasthenia were justified taking into account the use of immunosuppression, specific viral-neutralizing immunoglobulins and massive IgG immunoglobulin therapy, and the application of anti-inflammatory recombinant interleukins.

Keywords: clinical myasthenia phenotypes; autoantibody spectrum; regulatory T lymphocytes; co-stimulating molecules; acute phase proteins

Introduction

Nowadays, there is a worldwide steady increase in autoimmune diseases, among which myasthenia takes a special place. The disease is characterized by progressive muscular weakness; it is often accompanied by structural and functional changes in the thymus. The study of pathogenesis and treatment of myasthenia is an urgent problem due to an increase in incidence of the disease and its social significance, since the disease often occurs in young people of working age (20–40 years), and women are affected more often, with the course of the disease often being more severe compared with men (Berrih-Aknin et al., 2014; Tovazhnyanskaya & Samoylova, 2016).

The development of muscle weakness in the majority of patients is caused by disturbance of synaptic transmission in the neuromuscular synapses due to the presence of autoantibodies (AAbs) against nicotinic acetylcholine receptors (nAChR). This type of AAbs, according to various authors, is detected in 60–85% of patients examined for myasthenia (Carr et al., 2010; Phillips & Vincent, 2016).

However, autoantibodies AAbs to nAChR (nAChR AAbs) are not revealed in a large number of patients with myasthenia, and such patients are considered to be seronegative (SN) (Devic et al., 2014; Richard & Howard, 2017). SN patients have other mechanisms responsible for the myasthenia development – the presence of antibodies to the rianodine receptors, muscle-specific tyrosine kinase (MuSK), and to LPR4 protein associated with the low density lipoprotein receptor (Cavalcante et al., 2012; Burden et al., 2013; Shen et al., 2013; Skok, 2013; Vincent et al., 2018). Among the SN patients with generalized myasthenia, 40% have AAbs to muscle-specific tyrosine kinase (MuSK) (Huijbers et al., 2013; Koneczny et al., 2013). The binding of AAbs to nAChR of muscle cells is accompanied by the activation of complement proteins and the formation of a lysing complex that violates the structural integrity of the post-synaptic membrane and prevents the signal from passing through the nerve ending (Dedaev, 2014; Tovazhnyanskaya & Samoylova, 2016). Autoimmune processes in myasthenia can affect not only the neuromuscular synapses, but other organs and tissues of the body as well (thyroid gland, adrenal gland, spleen) (Jiang et al., 2013; Lopomo & Berrih-Aknin, 2017).

In the development of myasthenia, the thymus is obviously involved; there are both autoimmune cells and antigens located in it that facilitate their sensitization. According to Sprent (2002), normal expression of nAChR by thymus myoid cells plays an important role in inducing central immune tolerance to muscle proteins of the body (Sprent & Kishimoto, 2002). The certain subunits of AChR were expressed by thymus epithelial cells (Levinson, 2013; Makino et al., 2017; Nakamura et al., 2018). This mechanism is partially controlled by the autoimmune regulator AIRE (Berrih-Aknin, 2014). The regulator controls the representation of the nAChR peptides by the molecules of the main complex of histocompatibility (MHC) in the differentiation of T cells, which maintains immunological tolerance to the AChR (Sakaguchi, 2005; Dalakas, 2012; Ha & Richman, 2015).

The disease is often considered to be associated with the formation of thymus tumours (thymomas), and the generally accepted method of myasthenia treatment is the removal of thymus – thymectomy. However, surgical treatment for myasthenia is not always effective, and some pati-

ents experience a relapse of the disease after short-term remission (Klimova et al., 2016). The role of etiological factors of immune imbalance in myasthenia may be played by bacterial and viral infections in the thymus, which change the physiological activity of cells and contribute to the development of inflammation that induces the formation of myasthenia (Barzago et al., 2016). Clinical manifestations of myasthenia are heterogeneous in terms of severity, progression, localization of neurotransmitter disturbance, and the nature of the damage to the thymus. They are characterized by a wide range of symptoms – from ptosis in local forms to severe violations of respiratory functions in generalized forms of myasthenia.

In patients with MuSK AAbs, clinical manifestations of myasthenia are non-typical in comparison with patients having nAChR AAbs, and the debut of the disease is accompanied by facial, bulbar manifestations, and weakness of the neck muscles; patients have a marked atrophy of the muscles with relative preserved work of eye muscles (Phillips & Vincent, 2016; Galassi et al., 2018).

Although complex pathogenetic therapy of myasthenia, besides thymectomy, includes a wide range of agents, such as anticholinesterase drugs, immunosuppressive and cytostatic agents and methods of extracorporeal hemocorrection, the clinical effect of treatment is not always attainable. To improve the results of therapy it is necessary to substantiate the choice of approach to individual treatment, including surgical options, using various methods of correction of the whole complex of metabolic and immunological disorders.

Autoimmune reactions in myasthenia may be the result of loss of central or peripheral self-tolerance. The mechanism of central self-tolerance loss lies in the violation of the negative selection of B lymphocytes in the bone marrow and T lymphocytes in the thymus that leads to the formation of aggressive clones of B and T lymphocytes, and AAbs (Bach, 2012). Mutations of immunoglobulin genes, the existence of cross-reactions of autoantigens with antigens of microorganisms, and sometimes just an excessive increase in the concentration of autoantigens as a result of trauma or other destructive processes leads to the awakening of sleeping autoimmune clones and the loss of peripheral self-tolerance. An important role is also played by so-called regulatory T lymphocytes ($CD^{4+}CD^{25+}$), which provide suppression of immune response to their own antigens (Dyachenko et al., 2014; Aricha, 2016; Danikowski et al., 2017). The understanding of individual immune reactions responsible for the disease development in each particular case is essential for the choice of optimal approach to myasthenia treatment (surgical intervention, immunosuppressive therapy or plasmapheresis).

The objective of our study was to substantiate an individual approach to the treatment of various clinical forms of myasthenia by study of mechanisms and markers of loss of central and peripheral self-tolerance in thymus-independent myasthenia and thymus-dependent myasthenia gravis on the background of thymus hyperplasia and thymoma.

Materials and methods

We examined 427 patients with different clinical myasthenia phenotypes. They were classified into three groups according to the structural and functional changes in the thymus and the age. The first group included 62 patients with myasthenia without thymus affection with an average age of 43 years (M). The second group included 238 patients with myasthenia against the background of thymus hyperplasia (MH) with an average age of 30 years. The third group included 127 patients with myasthenia against the background of thymoma and an average age of 45 years (MT). The components of the patients' blood (blood cells and serum) served as materials for study.

The content of autoantibodies (AAbs) against the α_1 and α_7 subunits of nAChR in patients with different clinical phenotypes of myasthenia was determined by the method of immunoassay using as the antigens the recombinant extracellular domains α_1 (1–208) and α_7 (1–208) kindly provided by Prof. S. Tzartos (Gergalova et al., 2011). The results obtained were expressed in units of optical density at a wavelength of 490 nm. The concentration of AAbs to other antigens was determined by the enzyme-linked immunosorbent assay using the Eli-Viscero-Test kits ("Immunkulus", Russia) (Poletaev, 2008).

The concentration of circulating immune complexes (CIC) and the CIC constant (CIC-c) was determined by the spectrophotometric method according to the degree of precipitation in PEG6000 (Sergeeva, 1999).

The levels of complement C_3 and C_4 components were determined by the immunoturbidimetry method (sets of FENOX Medical Solution, Belarus) (Nilsson & Nilsson, 2012).

The concentration of acute phase proteins, ceruloplasmin and haptoglobin, was determined in blood serum by spectrophotometry using the Ravin method and with rivanol, respectively (Kamyshnikov, 2004).

The content of the C-reactive protein (CRP) was determined by agglutination in a latex-test. Quantitative determination was performed by multiple dilutions of blood serum and repeated agglutination reactions (Kamyshnikov, 2004).

Functional activity of neutrophilic granulocytes of peripheral blood was evaluated by light microscopy according to phagocytic index (PI), phagocytic number (PA) and index of phagocytosis completion (IPC) (Muniz-Junqueira et al., 2003). The oxidation-reducing activity of neutrophils was evaluated by light microscopy in NBT-test (Park et al., 1968).

The cytofluorimetric analysis of the lymphocyte population, the expression of activation markers was performed 2 hours after blood sampling, using test tubes with the K3 EDTA according to the standard protocol. In each sample, at least 10.000 cells were analyzed. Monoclonal antibodies against CD^4–PE, CD^{25}–FITC, and CD^{28}–FITC manufactured by "Beckman Coulter", USA, were used. For correct exclusion from the analysis zone of cells that did not meet the parameters, the necessary logical constraints were introduced into the particle distribution histogram for low-angle, side scintillation (SSC). The evaluation of expression level of surface receptors was performed at a mean intensity of fluorescence (MFI). To remove erythrocytes, the sample were prepared using no-wash technology OptiLyse C (Beckman Coulter, USA) was used. The analysis of stained cells was carried out on a flow cytometry Cytomics FC500 (Beckmann Coulter, USA).

The evaluation of the functional state of peripheral blood lymphocytes was determined by their proliferative activity in cell culture without additional stimulation and under the influence of the mitogen phytohemagglutinin (PHA). The assessment of the results of lymphocytes blast transformation was performed by light microscopy (Khaitov & Ilina, 2009).

Concentration of IL-1β, IL-2, IL-4, in the serum was determined by solid-phase immunoassay test systems using specific monoclonal antibodies (mAbs) to IL-1β, IL-2, IL-4 ("Vector-Best", Ukraine), sorbed on polystyrene plates, and horseradish peroxidase as an indicator enzyme.

The statistical processing of the obtained results was performed by Statistica 6.1 program. To compare average values ANOVA was used, with values $P < 0.05$ considered statistically significant. Experiment data is presented as ($x \pm SE$).

Results

One of the main pathogenetic factors of autoimmune myasthenia is the presence of AAbs to nAChR (Masuda et al., 2012). Our study of such antibodies in serum of myasthenia patients revealed their high concentration in all examined groups in comparison with the control, maximum concentration of AAbs to α1 subunit of MH group (Fig. 1). By contrast, the level of antibodies to the neuronal type of the α_7 subunit of nAChR was lower than the reference values of healthy people in all groups; the lowest value was observed in the MH group. Thus, in patients with myasthenia the occurrence of AAbs to muscular form of nAChR was observed in all cases that, probably, had caused the manifestation of clinical symptoms of muscle weakness. In the group of patients with myasthenia without thymus involvement and with early debut disease the AAbs content to α_7 subunit nAChR was elevated.

Since autoimmune processes in myasthenia are relevant not only to the muscular system, we have investigated the content of serum AAbs to a wide range of antigens. Patients of the M group showed a signifycant increase in the relative content of AAbs to β_2-glycoprotein I 56.4 ± 12.6%, to the cytoplasmic antigens of the renal tissue 64.7 ± 0.4%, to thyroglobulin 51.5 ± 13.8%, also to DNA 30.6 ± 1.9%, AAbs to the TSH receptor 38.4 ± 6.5%, AAbs to membrane antigens of the mucous

membrane of the stomach 31.9 ± 5.3% and the small intestine 49.4 ± 2.9% of the individual average level of immunoreactivity of the examined patients (Fig. 2).

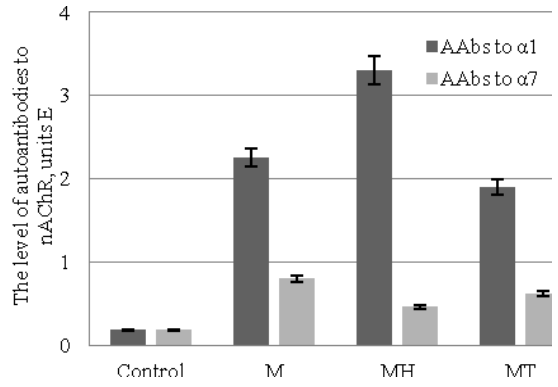

Fig. 1. The level of antibodies to α_1 and α_7 subunits of nAChR in patients with different clinical myasthenia phenotypes: M – myasthenia without thymus affection (n = 23); MH – myasthenia with thymus hyperplasia (n = 28); MT – myasthenia with thymoma (n = 31); control group n = 10

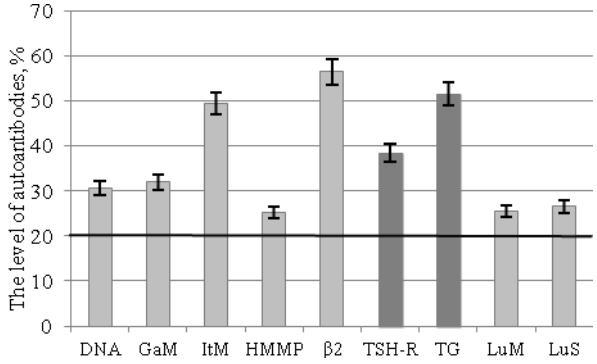

Fig. 2. Characteristic spectrum of autoantibodies (AAbs) in patients with thymus-independent myasthenia (M, n = 35): DNA – AAbs to DNA; GaM – AAbs to antigens of the gastric mucosa; ItM – AAbs to small intestinal mucosa antigens; HMMP – AAbs to mitochondria; β_2 – AAbs to β_2-glycoprotein I; TSH-R – AAbs to the TSH receptor; TG – AAbs to thyroglobulin; LuM – AAbs to membrane antigens of lung tissue; LuS – AAbs to cytoplasmic antigens of lung tissue; horizontal line shows control level

Patients of the MH group showed a significant increase in AAbs to TSH receptor 84.7 ± 7.9%, and to thyroglobulin 119.6 ± 36.4%, as well as increased AAbs to mitochondria of hepatocytes 25.9 ± 0.2%, to β_2-glycoprotein I – 30.6 ± 0.3%, to the protein of the astrocyte intermediate filament – 34.3 ± 0.3%, and to insulin – 29.6 ± 0.2% (Fig. 3).

The study of the relative content of AAbs (as a percentage of the individual average) showed that in the MT group the content of AAbs to DNA was 34.9 ± 2.4%, to the membrane antigens of the gastric mucosal cells – 35.5 ± 4.8%. The concentration of AAbs to β_2-glycoprotein I was two times as much as that of control and amounted to 49.4 ± 11.4%, to cytoplasmic antigens of the pulmonary tissue – 43.3 ± 9.8%, to thyroglobulin – 41.4 ± 2.6%, to the TSH receptor – 35.0 ± 5.1%, to hepatocyte mitochondria – 27.4 ± 1.8% (Fig. 4).

In the M and MT group, the AAbs spectrum was rather broad and included 12 specificities out of 24 examined. In contrast, in the MH group an increase in AAbs was found against only 6 specificities out of 24. In all three groups, AAbs were detected with high frequency to the TSH receptor (in the M group with a frequency of 55.6%, in the M group – 66.7%, in the MT group – 70.0%), to thyroglobulin (in the M group – 33.3%, in the group MH – 66.7%, in the group MT – 40.0%), to β_2-glycoprotein I (in the group M with a frequency of 33.3%, in the group MH – 33.3%, in the group MT – 20.0%) and against mitochondria (in the M group with a frequency of 55.6%, in the group MH – 33.3%, in the group MT – 10.0%). In addition, 55.6% of M group patients showed AAbs to DNA, TSH receptor, and membrane antigens of hepatocyte mitochondria; 44.4% of patients had AAbs to the membrane antigens of the mucous membrane of stomach and small intestine; 22.3% – to membrane antigens of the lung tissue and myocardial cells. There was an increase in AAbs to the S100 protein, cytoplasmic liver antigens, and cytoplasmic pulmonary antigens.

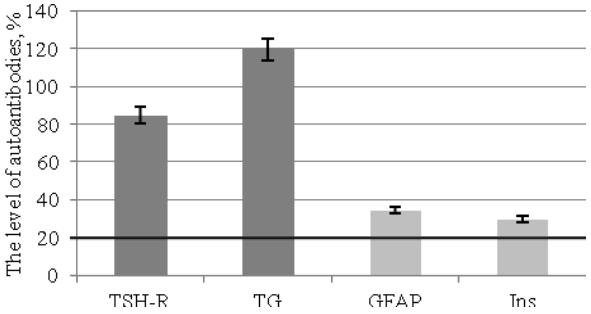

Fig. 3. Characteristic spectrum of autoantibodies (AAbs) in patients with thymus-dependent myasthenia (MH, n = 35): TSH-R – AAbs to the TSH receptor; TG – AAbs to thyroglobulin; GFAP – AAbs to astrocytes; Ins – AAbs to insulin; horizontal line shows control level

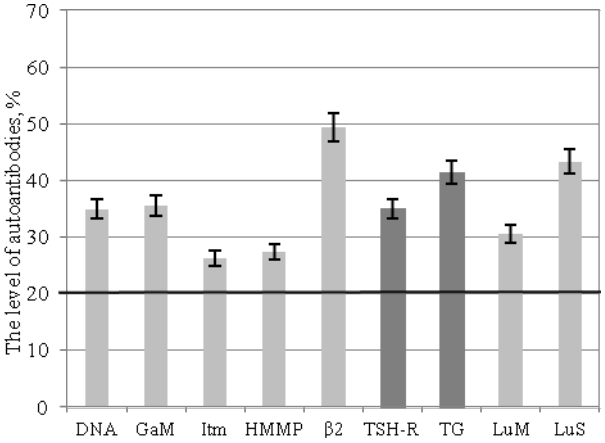

Fig. 4. Characteristic spectrum and concentration of autoantibodies (AAbs) in patients with thymus-dependent myasthenia (MT, n = 47): DNA – AAbs to DNA; GaM – AAbs to antigens of the gastric mucosa; ItM – AAbs to small intestinal mucosa antigens; HMMP – AAbs to mitochondria; β_2 – AAbs to β_2-glycoprotein I; TSH-R – AAbs to the TSH receptor; TG – AAbs to thyroglobulin; LuM – AAbs to membrane antigens of lung tissue; LuS – AAbs to cytoplasmic antigens of lung tissue; horizontal line shows control level

In 40% patients of the MT group, AAbs to DNA were detected, in 30% – to membrane antigens of the gastric mucosal cells, and in 20% – to membrane antigens of the mucous membranes of the small intestine. Besides, in this group AAbs were also found to other antigens, such as membrane antigens of hepatocyte mitochondria, protein of astrocyte intermediate filament, membrane and cytoplasm antigens of the pulmonary tissue, and to specific protein of axon myelin sheaths.

Thus, in all groups of patients with myasthenia we observed autoimmune reaction, the specificity of which was somewhat different between the groups. In patients with thymus hyperplasia the reaction was mostly directed against the muscle α1 subunit of nAChR; in other groups AAbs were found against a wide range of antigens, which can be considered as a potential factor of disorders in the respective organs and systems.

One of the mechanisms of pathogenic action of AAbs is their participation in the formation of circulating immune complexes (CIC), which, on the one hand, are functioning as a form of elimination of antibodies from the bloodstream, and on the other hand, – as an effective means of activation of immune cells Concentration of CIC was elevated in all studied groups, the CIC constant was decreased in all groups by 20% an average (Table 1).

Table 1
The content of circulating immune complexes (CIC) and their constants (CIC-c) in patients with different clinical phenotypes of myasthenia

Indicators	Control, n = 25	Study groups (x ± SE)		
		M, n = 62	MH, n = 238	MT, n = 127
CIC, units E	98.3 ± 21.1	148.3 ± 11.4*	152.6 ± 8.1*	186.0 ± 8.9*
CIC-c, CU	1.31 ± 0.04	1.06 ± 0.02	1.05 ± 0.01	1.04 ± 0.06

Note: M – myasthenia without thymus affection; MH – myasthenia with thymus hyperplasia; MT – myasthenia with thymoma; * – the differences are reliable relative to the control, P < 0.05.

CIC are potent activators of natural immune cells, which express receptors to Fc fragments of immunoglobulins of monocytes, macrophages, and neutrophils. The study of oxygen-dependent processes of phagocytosis in the NBT-test revealed the intensification of spontaneous reactions of oxidative metabolism in neutrophils by active forms of oxygen (AFO) in all patients with different clinical phenotypes of myasthenia; it was proved by elevated values of the spontaneous NBT-test in all groups (Table 2).

Table 2
Metabolic redox reserve of neutrophilic granulocytes in patients with different clinical phenotypes of myasthenia

Indicators of the NBT test	Reference values	Study groups (x ± SE)		
		M, n = 62	MH, n = 238	MT, n = 127
NBT test, spontaneous, %	10.0 ± 1.1	37.5 ± 5.2*	44.5 ± 5.6*	43.6 ± 6.1*
NBT test, stimulated, %	57.5 ± 3.0	63.1 ± 8.2	65.4 ± 5.9	63.0 ± 7.8
ACK spontaneous (CU)	1.50 ± 0.32	0.61 ± 0.09*	0.70 ± 0.30*	0.83 ± 0.32*
ACK stimulated (CU)	1.50 ± 0.21	1.13 ± 0.09	1.09 ± 0.32	1.00 ± 0.11
Stimulation index	7.50 ± 0.90	1.82 ± 0.43*	1.39 ± 0.60*	1.64 ± 0.30*

Note: see Table 1.

However, the stimulated NBT test indicated a decrease in the functional reserve of the oxygen mechanism of bactericidal effects of phagocytes, as evidenced by the decreased values of the stimulation index, the most pronounced in the MH group. It characterizes the high degree of activation of intracellular NADP-H-oxidase activity. Thus, neutrophils of patients with myasthenia were in pre-activated condition possibly due to the presence of CIC, but they were less responsive to stimulation.

The lowest index of stimulation was found in the group of patients with thymic hyperplasia (MH).

Consequently, in all patients with different forms of myasthenia the various degrees of increase in oxidative reactions were revealed at the expense of the AFO. The oxidative reactions in the stimulated by zymosan NBT-test were higher, but the differences in the groups were insignificant. Alongside this, the oxygen-independent phagocytosis index (PI) of neutrophils in myasthenia patients was also lowered in comparison with the reference values (Table 3); the most pronounced decrease was recorded in the MH group.

Table 3
Phagocytic activity of neutrophilic granulocytes in patients with different clinical phenotypes of myasthenia

Indicators of phagocytic activity of neutrophils	Reference values	Study groups (x ± SE)		
		M, n = 62	MH, n = 238	MT, n = 127
Phagocytic index,%	85.0 ± 5.1	82.7 ± 7.9*	74.0 ± 6.4*	81.7± 8.5*
Phagocytic number	3.20 ± 0.21	3.33 ± 0.90	2.85 ± 0.60	3.51 ± 0.90
Index of completeness of phagocytosis	1.85 ± 0.12	1.14 ± 0.22	1.06 ± 0.08	1.03 ± 0.19

Note: see Table 1.

Violation of the primary barrier function of the immune system in myasthenia determines reduced resistance to viral and bacterial infections; it is a significant factor for the debut and the progression of muscle weakness. In all our patients with different clinical forms of myasthenia, disorders of functional activity of peripheral blood neutrophils was revealed. Disorders were characterized by inadequate digestibility of phagocytic reactions, and reduced metabolic reserve of oxygen-dependent enzyme systems revealed in the NBT-test, which are likely to be due to high degree of cell sensitization and depletion of the reserve of intracellular processes involving active forms of oxygen. The most pronounced violation was found in the group of patients with thymus hyperplasia (MH).

Natural immune cells are producers of acute phase proteins: C-reactive protein (CRP), haptoglobin, ceruloplasmin, and the complement C_3 and C_4 components, which are powerful inflammation factors. The study of acute phase proteins in serum of patients in all groups showed a significant (6–9 times) increase in the levels of CRP especially in the MH group (Table 4).

Table 4
The level of acute phase proteins in patients with different clinical phenotypes of myasthenia

Indicators	Reference values	Study groups (x ± SE)		
		M, n = 62	MH, n = 238	MT, n = 127
Haptoglobin, g/L	1.09 ± 0.27	1.66 ± 0.24	1.24 ± 0.46	2.97 ± 0.77
Ceruloplasmin, mg/L	315.0 ± 45.0	209.3 ± 18.6*	138.3 ± 8.5*	267.0 ± 23.2*
C-reactive protein, mg/L	3.0 ± 2.8	14.8 ± 8.2*	25.7 ± 7.9*	20.8 ± 5.7*

Note: see Table 1.

An increase in haptoglobin was observed in patients of the MT and, to a lesser extent, the M group; the level of ceruloplasmin was even slightly lower compared to control. The levels of C_3 component in the M group did not much differ from the control values (Fig. 5a). In the MT group, the average concentration of the C_3 component was also significantly higher than in control.

The concentration of the C_4 component that mediates the antibody-dependent complement activation pathway was decreased in the M and MT groups (Fig. 5b).

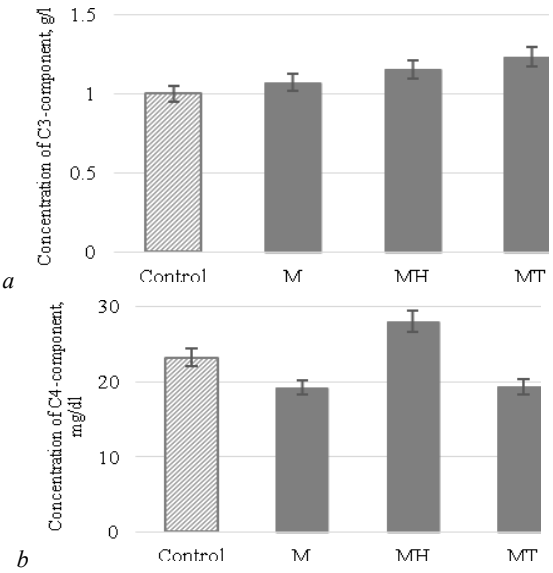

Fig. 5. Concentration of the C_3 (*a*) and C_4 (*b*) component of complement in patients with different clinical phenotypes of myasthenia gravis: M – myasthenia without thymus affection (n = 44); MH – myasthenia with thymus hyperplasia (n = 95); MT – myasthenia with thymoma (n = 67); P < 0.05 in comparison with the control

CRP is one of the key components of humoral natural immunity that provides a connection between congenital and adaptive immune responses. Its increase in all groups of patients is consistent with high levels of AAbs in blood serum of these patients. The maximum values of CRP and antibodies to muscle nAChR were found in the MH group. Thus, the main proinflammatory factor in myasthenia was CRP, the level of which was maximally elevated in the MH group (Table 4); so it is the MH group in which the most aggressive autoimmune reaction against muscle nAChR can be expected (Tzartos & Lindstrom, 1980; Bach, 2012).

Production of AAbs depends on the activity of cells of adaptive immunity – T and B lymphocytes. In humans, the cells most available for study are peripheral blood lymphocytes, most of which belong to T

lymphocytes. We determined the proliferative activity of peripheral blood lymphocytes in patients with myasthenia in a spontaneous and PHA stimulated reaction of blast transformation, the results of which are presented in Table 5. The data show a high level of proliferative activity of peripheral blood lymphocytes on 48-hour cultivation (without mitogenic stimulation) in patients of groups M and MH. In patients of the MT group spontaneous proliferative activity did not differ from control. Stimulation with PHA resulted in a strong proliferative response in the MT group, a marked response in the M group, but a very weak reaction was in the MH group.

Table 5
Proliferative activity of peripheral blood lymphocytes in cell culture

Indicators	Reference values	Study groups (x ± SE)		
		M, n = 62	MH, n = 238	MT, n = 127
Number of blast cells without mitogen stimulation,%	11.0 ± 2.5	22.0 ± 9.8	22.4 ± 3.2*	12,0±3,1
Number of blast cells stimulated by mitogen PHA,%	45.5 ± 4.5	53.0 ± 7.0	32.5 ±2.6	39.6 ± 1,6
Index of stimulation	2.60 ± 1.30	1.40 ± 0.61	0.45 ± 0.32*	5.19 ± 0.19*

Note: see Table 1.

Thus, myasthenia without thymus damage (M) was accompanied by a preactivated condition of peripheral blood T lymphocytes that retained the ability to be activated by mitogen. In patients with thymus hyperplasia (MH), T lymphocytes were also in a preactivated condition, but they were significantly less responsive to mitogenic stimulation. In patients with thymoma (MT), T lymphocytes were not preactivated, but they were potently responding to PHA activation.

Different mechanisms of self-tolerance loss can concern processes that occur both in the central organ of immunity, the thymus, and in the peripheral immune system (Sprent & Kishimoto, 2002; Pevzner et al., 2012). An important role in supporting self-tolerance belongs to regulatory T lymphocytes. In all examined patients, a significant decrease in the expression of markers of regulatory T cells CD^{4+} CD^{25+} against the moderate reduction of total CD^{4+} CD^{28+} T lymphocytes background was found.

The maximum decrease in CD^{4+} CD^{28+} co-stimulation markers compared with control was found in the group of patients with myasthenia without morphofunctional changes in the thymus (M) – 38.0 ± 1.6% versus 62.1 ± 5.8% (Fig. 6a). In the MH group, the reduction in CD^{4+} CD^{28+} markers was less pronounced – 46.7 ± 2.1%; in the MT group, the level of these markers was higher – 52.0 ± 4.4%.

The maximum reduction in the number of Treg CD^{4+} CD^{25+} cells was found in all groups of patients with myasthenia on the background of thymus hyperplasia (MH) – 0.6% vs. 3.2% in the control. In other groups, the number of regulatory cells was higher than the MH group, but still lower than in the control: 1.9% and 2.1% respectively in the groups M and MT (Fig. 6b). Thus, the maximum decrease in the number of regulatory T lymphocytes was revealed in a group of patients with thymus hyperplasia, namely those with the highest levels of antibodies to muscle nAChR (Fig. 1), which proves the essential role of regulatory cells in support of self-tolerance to nAChR.

In the study of the cytokine profile in patients with different clinical forms of myasthenia, decrease in the concentration of IL-1β in the MH and MT groups was found. The study of IL-4 content showed that this anti-inflammatory cytokine exceeded control values in MH and MT groups. The content of IL-2 was also significantly increased in these groups (Table 6).

Table 6
The cytokine level in patients
with different clinical phenotypes of myasthenia (x ± SE)

Indicators	Control, n = 25	Study groups		
		M, n = 62	MH, n = 238	MT, n = 127
IL-1β, pg/ml	1.60 ± 0.30	1.10 ± 0.60	0.51 ± 0.10*	0.50 ± 0.20*
IL-2, pg/ml	2.70 ± 0.40	6.60 ± 3.21*	11.40 ± 7.80*	15.30 ± 9.63*
IL-4, mmol/L	70.0 ± 22.9	337.3 ± 59.4	492.5 ± 52.8*	666.4 ± 44.5*

Note: see Table 1.

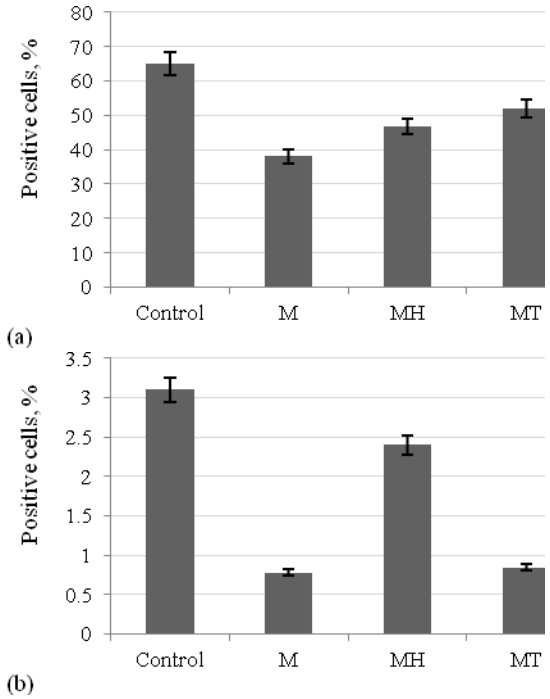

Fig. 6. The level of subpopulations of cells expressing CD^{4+} CD^{28+} (*a*) and CD^{4+} CD^{25+} (*b*) differentiation clones on lymphocytes in patients with different clinical myasthenia phenotypes: M – myasthenia without thymus affection (n = 22); MH – myasthenia with thymus hyperplasia (n = 38); MT – myasthenia with thymoma (n = 37); P < 0.05 in comparison with the control

The maximum value of IL-4 was found in patients with myasthenia against the background of thymoma (666.4 ± 44.5 mmol/L). In thymus hyperplasia against the background of myasthenia, a sevenfold increase in the content of IL-4 (492.5 ± 52.8 mmol/L) was revealed (Table 6).

Discussion

According to Cavalcanti et al. (2012), AAbs to nAChR localized on the postsynaptic muscular plate are major myasthenogenic factors in 80% of patients with myasthenia; in 10% of cases, AAbs are directed against a specific tyrosine kinase (MuSK) that participates in the clustering of AChR by the formation of the MuSK-LRP4-Agrin complex. The study by Tzartos & Lindstrom (1980) shows that in seropositive patients AAbs are formed into so-called "main immunogenic site" of nAChR, which is located in the extracellular part of the α-subunit (amino acid residues 67–76).

As was shown in the review by Melzer et al. (2016), neoplastic epithelial cells of the thymus expressed a large number of autoantigens, including unordered unrolled subunits of nAChR. In addition, the formation of regulatory T-lymphocytes is disturbed in patients with thymoma.

As a result, the activation of self-reactive clones of T and B lymphocytes and the production of AAbs to the nAChR of the muscular postsynaptic plate take place (Curnow et al., 2001; Darabid et al., 2014). Our findings are consistent with this mechanism. However, we observed the highest level of AAbs against α_1 subunit nAChR (more than double compared to control) in patients with hyperplasia of the thymus, but not with thymomas (Fig. 1). It is noteworthy that the levels of antibodies to another nAChR subtype ($\alpha_7$subunit) in patients with myasthenia were below the reference values of healthy people, indicating selective self-tolerance damage directed specifically against nAChR muscular form. Alongside this in the groups M and MT, a wide range of AAbs was found including AAbs to antigens of 12 specificities (DNA, mitochondria, β_2-glycoprotein I, the cell membrane of the intestine, lungs, and liver); and in the MH group AAbs range was limited to key antigens of 6 specificities (TSH, TH, β_2-glycoprotein I and mitochondria receptors) indicating the prevailing autoimmune reaction against muscle nAChR in these patients.

Decrease in the portion of CD^{4+} CD^{28+} T lymphocytes that determines the overall level of T dependent immune response supposedly contradicts the development of autoimmune response in patients with myasthenia. However, according to some authors, the interaction of CD^{28+} on T cells and B_7 in the AIC is important not only for the activation of effector T cells, but also for the expansion and maintenance of regulatory T lymphocytes CD^{4+} CD^{25+} (Sakaguchi, 2005; Hall et al., 2015; Danikowski et al., 2017). Thus, a decrease in the number of CD^{4+} CD^{28+} T lymphocytes in all studied groups of patients is also a factor in the loss of self-tolerance. In our previous study we had shown that in patients with thymomas and significant increase in the expression of CD^{4+} CD^{28+}, the content of IL-2 was maximal. In patients with MH, the lower expression of CD^{4+} CD^{28+} was detected in comparison with the MT group and a less pronounced increase in IL-2 content, apparently against the background of early debut and short duration of the disease.

The activation of T-regulatory CD^{4+} CD^{25+} leads to the synthesis of IL-2, which takes part in the formation of tolerance (Sakaguchi, 2005). In MH, additional mechanisms of destruction of own cells and tissues can be enzyme activation and increased concentration of acute phase proteins that leads to the destruction of basal and cellular membranes, disturbance of the DNA structure, causing the destruction of cells by generating AFO with phagocytes of granulocytic neutrophils (Klimova & Kalashnikova, 2016).

This was characteristic for patients of MH, who had an increased spontaneous level of AFO formation and an 8-fold increase in CRP. The complexes CRP that are fixed on the membranes of microorganisms and damaged cells cause the activation of the complementary cascade, namely the C_3 component, which was observed in patients of MH against the background of increased levels of CRP and C_4-component of the complement.

Our study revealed the decrease by 40%, in the level of the C_4 component of the complement in MT patients and in MH patients the component was increased. In patients with thymus hyperplasia, the activation of the complement cascade to 27.8 ± 3.2 g/l (with a control of 21.0 ± 0.35 g/l) was detected, which is consistent with the data of Masuda, who states that the activation of the complement leads to end plate destruction (Masuda et al., 2012).

In patients with thymus hyperplasia (MH), cells of both natural (neutrophils) and adaptive immunity (T lymphocytes) are in the state of maximum activation and do not respond to additional stimulation. The humoral immune response is directed mainly against muscle nAChR (Nakamura et al., 2018). In the blood of these patients, the maximum increase in the level of CRP is acting as opsonin through stimulation of phagocytosis and cells of the monocyte-macrophage system, which causes the general state of inflammation. Proteolytic products of CRP are regulators of T and B lymphocyte proliferation. Binding of C-reactive protein to T lymphocytes affects their functional activity by initiating the reactions of precipitation, agglutination, phagocytosis and complement fixation. The elevated level of the C_4 component of the complement observed in this group contributes to the cytotoxicity of autoantibodies and is probably due to the high concentration of CIC that activates the components of the complement system (Kusner et al., 2013).It should also be noted that in the MH group the minimal level of regulatory CD^{4+} CD^{25+} T lymphocytes was determined. In these patients the most pronounced manifestation of myasthenia symptoms can be predicted, but damage to other organs and tissues can be moderate.

In patients with thymoma (MT) the cells of natural immunity (neutrophils) are activated and the level of CRP is significantly increased. T lymphocytes in these patients are in a state of rest and respond normally to mitogenic stimulation. These patients have a relatively low level of AAbs to muscle nAChR, but there are AAbs against numerous antigens of other organs and tissues and there is the highest level of CIC. The maximum level of co-stimulating CD^{4+} CD^{28+} and regulatory CD^{4+} CD^{25+} molecules was found that may suppress other cells in inflammatory processes. In these patients more general autoimmune reaction with lesions of various organs and tissues can be expected.

In patients with myasthenia, without damage to the thymus (M), the cells of natural immunity are activated less than in two previous groups, and the level of CRP is also lower, although substantially higher compared to control. The level and specificity of AAbs and the number of immune complexes are similar to those in the MT group.

Conclusion

Summarizing the data obtained, it is possible to characterize the state of immune reactivity of various groups of patients with myasthenia as follows. Patients with thymus hyperplasia (MH) can be recommended for plasmapheresis to remove autoantibodies and immune complexes, as well as anti-inflammatory and immunosuppressive therapy. Most likely, in patients with thymoma (MT group), the development of myasthenia is provoked by a breakdown of peripheral tolerance (activation of "sleeping" autoimmune B-lymphocyte clones) as a result of infections or the appearance of tumour antigens. Probably, in this case thymectomy is justified, as it reduces the concentration of tumour autoantigens. In patients with myasthenia, without damage to the thymus (M) the lymphocytes are in the activated state, but they are able to respond to stimulation, that is, there is an active immune reaction. In these patients, activation of the adaptive immune system, including both B and T lymphocytes, is evident. Obtaining new knowledge about the mechanisms of self-tolerance loss will justify the choice of approaches to the treatment for various clinical myasthenia phenotypes, including thymectomy and specific types of treatment (anti-inflammatory therapy, hormone therapy, plasmapheresis, immunosuppression).

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