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Der Pharmacia Lettre, 2017, 9 [6]:8-17
[http://scholarsresearchlibrary.com/archive.html]

ISSN 0975-5071
USA CODEN: DPLEB4

On Forming Central and Peripheral Markers of Self-Tolerance Loss in Diverse Clinical Myasthenic Phenotypes

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ABSTRACT

Various variants of the immune response should be used to choose an appropriate approach to the complex treatment of myasthenia and thymus affections. When specific markers are modified, there should be selected an address correction of metabolic and immunological disorders. Surgical treatment is also expedient, when tyFigureal markers of central auto-tolerance loss are present, including the high concentration of auto-antibodies to viruses, phagocyte barrier malfunctions, an insignificant increase in antibodies to the subunits of acetylcholine receptors, the high concentration of proinflammatory cytokines, a low level

of regulatory CD4+CD25+cells. The preventative correction of humoral and cellular immunities will allow us to take preventative measures against complications (the auto-immune affection of other target organs) after surgical procedures (thymectomy and thymthymomectomy).

Key words: *Myasthenia, immunological modifications, surgical treatment.*

INTRODUCTION

Myasthenia is a serious autoimmune disease with antibodies disturbing a normal transfer of a neuromuscular impulse and thus causing slackening of load-dependent cross-striated muscles and abnormal fatigability. Moreover, myasthenia may often bring morpho-functional changes in the thymus. Diverse variants of classifying movement disorders and modified functions, characteristic of myasthenics, make it difficult to sort out clinical patterns of the disease. Therefore, it is important to understand pathogenetic mechanisms of both central and peripheral immune organs identifying the variety of clinical forms of the disease [1].

The mechanisms of forming central tolerance are known to control immune response against its own antigens in order to stop autoimmune aggression (by eliminating auto-reactive clones of T-cells in the thymus at early stages of its development). The development of peripheral tolerance is known to do the same thing with immune response with the help of energy by ignoring or suppressing the reaction to its own antigens. This energy is triggered by subclasses of CD4 T-lymphocytes, generated to suppress the so-called Treg cell auto-reactive T-lymphocytes [2].

So far, myasthenics have undergone an effective treatment. It has not been so in all cases, as standard treatment procedures include surgical removal of the modified thymus along with applying anti-choline-esterase medicines and hormones. Only some patients enjoy a considerable remission after thymectomy, whereas others, on the contrary, suffer myasthenic or cholinergic crises after the operation, as well as intensified auto-immune reactions as a result of attacks against nervous synapses and other target organs. Moreover, of some interest is the study of trigger factors of auto-immune reactions, the range of targets of generated auto-antibodies. In order to develop methods of target treatment of myasthenics, one should take into consideration central (in the thymus) and peripheral mechanisms of auto-tolerance loss [3]. Despite the fact that the range of medicines, used to treat generalized myasthenia and aimed at suppressing pathologic auto-immune processes, has been constantly expanding, it often happens that doctors fail to provide a sufficient therapeutic control for this serious disease. It confirms the idea of other activation mechanisms in existence and a maintained pathologic auto-immune process, namely, the interference of nervous,

endocrine, and immune systems. Diverse clinical implications, ambiguous assessments of separate myasthenic symptoms, no correlations between the seriousness in the presentations of the disease and electro-physiological modifications, as well as the probability of some concomitant pathology in existence affecting the auto-immune process, - this all turns into a burning problem of searching after new additional diagnostic and cure effectiveness control methods [4]. The purpose of the current work lies in studying peculiarities of immune-physiological reactions and auto-tolerance loss markers in the patients with various clinical myasthenic phenotypes.

MATERIALS AND METHODS

There were examined 208 patients (131 women and 77 men aged 13 through 70) with various clinical myasthenic phenotypes and structure functional modifications of the thymus. They were divided into three groups: the first one (M) consisted of 74 myasthenics without thymus affection; the second group (MG) included 92 myasthenics with thymus hyperplasia; the third group (MT) covered 42 myasthenics with various thymoma types.

The exploration concerned the barrier function of phagocyte granulocytic neutrophils; the persistency in herpetic viruses (cytomegalovirus, Epstein-Barr virus); the expression of CD25 differentiation clusters in activated lymphocytes; concentration changes in pro-inflammatory cytokines; the presence of specific auto-antibodies to subunits of nicotine acetylcholine receptors.

The phagocytal activity of granulocytic neutrophils was estimated with the help of the following three technical approaches:

1) depending on the ability of neutrophils to consume and digest *Saccharomyces cerevisiae* yeast by means of light microscopy. There was estimated the phagocytic index (PhI) – the number of cells, involved in phagocytosis; the phagocytic number (PhN) – the number of bacteria, consumed by one blood neutrophil; the index of phagocytosis completeness (IPhC) – the ability of neutrophils to digest [5];

2) digestibility and absorbability of neutrophils were assessed by the method of fluorescent microscopy with the application of the acridine orange dye (AO), taking into account that intact yeast cells are of green luminescence with constant intensity (λmax = 530 nm) while fluoro-chromium-plated by the dye. In the incubation, the green luminescence of the consumed yeast turns yellow, whereas in the complete digestion (in 45 minutes) it turns bright orange (λmax = 640 nm) thanks to DNA denaturation during its transformation from the double-spiral into unispiral form. As a rule, in an hour and a half digestion is followed by the complete lysis of the yeast. Actively digestible yeast was characterized by red luminescence [6].

3) the general enzymatic oxidation-reduction activity of neutrophils was estimated by the nitro blue tetrazolium restitution test (NBT-test). Light microscopy helped to discern blue violet diformasan granules in the phagocyte that corresponds with the sufficient activity of NADF-H-oxidase. There were estimated spontaneous (while applying a NBT dye) and stimulated (by adding a NBT dye and opsonizated zymosan) reactions. The level of antigen activity of non-activated neutrophils was evaluated by calculating the percentage of positive cells, which had absorbed the NBT dye (SP spontaneous reaction, ST stimulated reaction); the activation level of its intracellular enzymatic systems was estimated by calculating the average cytochemical coefficient during the spontaneous enzymatic activation (ACC SP) and stimulated enzymatic activation (ACC ST). There was assessed a stimulation index (SI) as a relation of absorbing cells in the spontaneous test (SP) to the number of phagocytes, which had absorbed the dye in the ST-NBT-test [7].

The expression of the CD25+ differentiation cluster in activated lymphocytes was evaluated by the method of indirect immune-fluorescence with the application of monoclonal anti-bodies, marked by the FITC dye. Dyed cells were analysed through luminescent microscopy. IL-2, IL-4, IL-8 and IL-10 concentration in blood serum was calculated with the help of the test system to hold an enzyme-linked immunosorbent assay using monoclonal anti-bodies, adsorbed in polystyrene plates. The generated "anti-gen-anti-body" complex was detected with the help of the conjugate, with its peroxidase catalyzing substrate splitting (of hydrogen peroxide) and changing the color of the indicator. Optical density was measured with the 450nm wave length in the StatFax 3200 immune-enzyme analyzer [8].

Specific antibodies to α1- and α7-subunits of nicotine acetylcholine receptors (nAChR) in blood serum were identified with the help of the test system for immune-enzyme analysis in a solid-phase carrier. The method is based on the specific interaction of receptor subunits with auto-antibodies to α1- and α7-nAChR subunits, contained in the sera under research. The number of auto-antibodies to α1- and α7-nAChR was judged by the measured optical density with the 450wave length in the StatFax 3200 immune-enzyme analyser [9].

RESULTS AND DISCUSSION

The research of the barrier phagocyte function was held with the help of the methods, which made it possible to see all phagocytosis stages, including chemotaxis, adhesion and endocytosis, and explore the intensity and speed of the digestion ability of intracellular activated ferments in phagocyte neutrophils.

The first method of visual assessment of chemotaxis, adhesion and endocytosis allowed us to evaluate the overall number of phagocytes in the patients with various clinical myasthenic phenotypes. The maximum number of the cells, involved in

phagocytosis (phagocytic index PhI), could be observed in M group (82,7 ± 7,9) %. The phagocytosis efficiency was judged by the final digestion ability of neutrophils (index of phagocytosis completeness IPhC). This indicator was lower than the control on average by 42% in all groups under research (see table 1). The group with MT (1,04 ± 0,21) showed the minimum value of endocytosis in neutrophil granulocytes.

Table-1: The phagocytic activity of granulocyte neutrophils in patients with various clinical myasthenic phenotypes

Indicators	Control	M	MG	MT
PhI, %	85 ± 5,1	82,70 ± 7,9*	77,04 ± 8,1*	81,71± 8,5 *
PhN	3,2 ± 0,2	3,33 ± 0,9	2,85± 0,6	3,51± 0,9
IPhC	1,85 ± 0,12	1,12 ± 0,4	1,05± 0,2	1,04± 0,21
Note: * - reliable with control, p ≤0,05				

If neutrophil granulocytes with undigested antigens migrate to various fibres of the organism, the deficient digestion ability of neutrophils may lead to negative consequences. The dynamics of the phagocytic activity was estimated taking into account the intensity of DNA denaturation of the microbes, seized by phagocytes. This procedure helped to visualize all the stages of endocytosis step by step. The green glowing, tyFigureal of native nucleic acids, linked with acridine orange (Figure1, Figure 1a) tended to change, when the dye was bonded with the denatured DNA of *S. cerevisiae*. Meanwhile, the fluorescent color turned red (Figure 1, Figure 1b), with the intensity of the red luminescence characterizing various functional stages of the activity of lysosomal enzymes of neutrophils (Figure.1, Figure 1b, 2b). Double-helical DNA transforming into unihelical showed that the anti-gen was intensively digested by the lysosomal enzymes of neutrophils, whereas undigested anti-gens preserved green luminescence of the native DNA (figure 1, Figure 2a).

AO coloring (λmax = 530 nm) AO coloring (λmax = 640 nm)

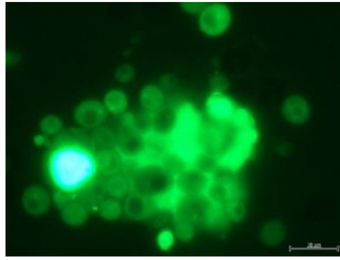

Figure 1a

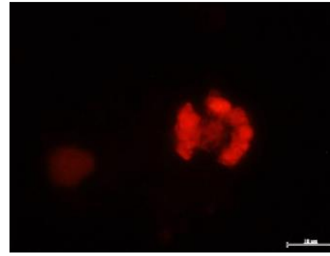

Figure1b

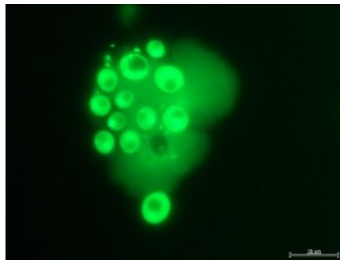

Figure 2a

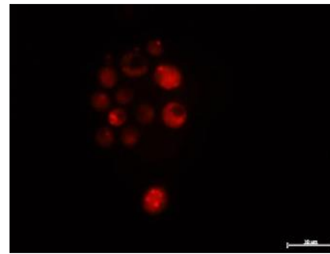

Figure 2b

Figure-1: Stages of DNA denaturation in *S. cerevisiae* at different phases of phagocytosis of granulocytic neutrophils in the patients with M (Figure 1a, 1b) and MT (Figure 2a, 2b)

Moreover, this method (the same as the visual assessment of phagocytosis) allowed us to see the lowest digestion ability of granulocytic neutrophils in the patients with MT: to be more specific, the yeast, which could not be digested, kept green luminescence and slight orange coloring (figure 1, Figure 1a, Figure 2a). Thus, while studying the peculiarities of antigen processing, phagocytosis completeness and functional activity of neutrophil enzymes, we revealed various levels of the barrier malfunction in phagocytes.

The NBT-test assessment of the neutrophil phagocytic activity showed that when with zymosan and influenced by neutrophil enzymes, active oxygen forms were generated with low intensity in all the myasthenics under research. Meanwhile, the number of formasan-positive cells (SP) in the spontaneous NBT-test was on average four times more than in control. This fact testifies a low oxidation reserve in exogenic enzymatic activation. This indicator (SP) presented the maximum increase by 4,4 times in MG and MT. Apart from that, there was observed a significant reduction in the average cytochemical coefficient (ACC SP) in the spontaneous NBT-test. The maximum reduction in the ACC SP could be seen in M group (at 30 %) due to the modified enzymatic oxidation-reduction activity (table 2). The stimulation index (SI) was significantly reduced in all the groups under

research by 3,5 times on average (with the lowest one in the MG group). This fact shows deficit of NADF-N-oxidase system of phagocytes in myasthenics, resulting in lowered endocytosis and the completeness of phagocytosis in neutrophils.

Table-2: Metabolic oxidation-reduction reserve of neutrophil granulocytes in the patients with various clinical myasthenic phenotypes

NST-test indicators	Control	M	MG	MT
SP, %	10 ± 1,1	39,23 ± 4,3*	44,48 ± 5,6*	43,62 ± 6,1*
ST, %	57 ± 3,2	62,69 ± 7,2	65,39 ± 5,9	63,03 ± 7,8
ACC SP, standard unit	1,5 ± 0,3	0,62 ± 0,08*	0,71 ± 0,3*	0,82 ± 0,31*
ACC ST, standard unit	1,5 ± 0,2	1,11 ± 0,2	1,11 ± 0,4	1,00 ± 0,1
SI	7 ± 0,9	1,82 ± 0,4*	1,39 ± 0,6*	1,64 ± 0,3*
Note: * - reliable with control, p ≤0,05				

The deficient phagocytic function of neutrophils points to the barrier malfunction of inborn immunity in all the clinical myasthenic phenotypes. All patients from the three groups (M, MG and MT) happened to have virus persistence: high G-class auto-antibody titers to cytomegalovirus and Epstein-Barr virus. Along with herpetic virus, the patients with M and MT often (36 %) happened to have auto-antibodies to virus B and virus type C hepatitis.

CD25+ activated lymphocytes in the patients with various clinical phenotypes of myasthenia. CD25 receptor, expressing in T and B-lymphocytes, functions as a ligand to IL-2 and marks the activation of immunocompetent cells. There was detected a slight change in the expression of the activated CD4+CD25+ lymphocytes in patients with various myasthenic phenotypes (table 3).

Table-3: The content of activated CD25 + lymphocytes in patients with myasthenia

Indicator	Control	M	MG	MT
CD25+	12,0±3,1	15,0±5,7	26,0±2,1*	19,0±3,4
Note: * - reliable with control, p ≤0,05				

The study of cytokine concentration in the patients with various clinical myasthenic phenotypes showed its considerable increase in the experimental groups. The proinflammatory IL-2 exceeded the control value in all the groups. The maximum increase of 5,7 times in the concentration of the above-mentioned interleukin was observed in the MT. This very group demonstrated tenfold increase in IL-4 and sixty-fold increase in IL-8. The MG group showed the maximum increase in IL-10 (table 4).

Table-4: The content of cytokines in patients with myasthenia the background of morpho-functional modifications in the thymus gland

Indicator	Control	M	MG	MT
IL-2, pg/ml	2,7 ± 0,4	6,6 ± 3,2*	11,4 ± 7,8*	15,3 ± 9,6*
IL – 4, мillimole/l	70,0 ± 22,9	337,3 ± 59,4	492,5 ± 52,8*	666,4 ± 44,5*
IL – 8, pkg/ml	10,0 ± 8,4	72,3 ± 21,5*	88,3 ± 34,2*	590,9 ± 67,1*
IL-10, pkg/ml	5,2 ± 0,8	1,0 ± 0,6*	16,6 ±8,8*	5,5 ± 0,9
Note: * - reliable with control, p ≤0,05				

While studying the presence of auto-antibodies to α1-nAChR, we saw the high level of their concentration in all the three groups, surpassing the control value by more than 1,5 times. The maximum increase in the concentration of auto-antibodies to α1-nAChR was observed in the MG group (by 2,2 times). The auto-antibodies to α7-nAChR exceeded the refer from hyperplasia (table 5).

Table 5: The content of antibodies to nAChR α1- и α7-subunits in patients with various clinical phenotypes of myasthenia

Indicator	Control	M	MG	MT
AB to α1 nAChR, unit of optimum density.	0,180 ± 0,43	0,373 ± 0,052	0,389 ± 0,065	0,281 ± 0,027
AB to α7 nAChR, unit of optimum density	0,180 ± 0,37	0,343 ± 0,089	0,268 ± 0,034	0,198 ± 0,021
Note: * - reliable with control, p ≤0,05				

There is a great number of peripheral tolerance mechanisms without immune reaction to auto-antigens, removing auto-aggressive clones with the help of apoptosis. Moreover, there can appear anergy to antigen stimulation thanks to changes in the activity of regulatory suppressing cells, which are crucial in the understanding of the regulating net of responses, maintaining homeostasis [1]. Regulatory cells, maintaining mechanisms of immunologic tolerance, are classified according to their background: natural (constitutive) and inducible (adaptive) regulatory CD4+CD25+ cells. Adaptive regulatory T-cells are divided into the following subtypes: the cells, which can produce IL-10 (these cells are likely to exist in myasthenics with MG), and cells producing IL-4. Adaptive regulatory cells and peripheral T-cells are often of the similar phenotype. Antigen-specific cell clones are found in the patients, infected with Epstein-Barr virus. Peripheral T-helper CD4-cells can be induced both as IL-10 and IL-4. Regulatory suppressing CD4+CD25+ cells play a significant role in the inhibition of other cells with infections. Moreover, regulatory cells of CD4-population are characterized by the constitutive CD25+ expression of IL-2α receptor [2]. The activation of regulatory cells leads to intensified synthesis of IL-2, participating in the formation of tolerance in the thymus and other anti-inflammatory cytokines.

CONCLUSION

In the future, the analysis of immune response variants can be used to choose an appropriate approach of complex treatment in case of myasthenia and various thymus affections. When specific markers are modified, there should be selected the address correction of metabolic immunological disorders. Surgical treatment is also advisable in case of figural markers of central auto-tolerance loss, including the high concentration of auto-antibodies to viruses, barrier phagocyte malfunctions, the insignificant increase in antibodies to subunits of acetylcholine receptors, the high concentration of proinflammatory cytokines, the low level of regulatory CD4+CD25+ cells. The preventative correction of the modified humoral cell immunity will make it possible to prevent complications (the auto-immune affection of other target organs), following surgical procedures (thymectomy and thymthymomectomy).

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