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Status of cellular immunity in rats under conditions of acute widespread petitonitis in the setting of diabetes mellitus

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ABSTRACT

Acute generalized peritonitis (AGP) occurs in patients with concomitant pathology, in particular at diabetes mellitus. The severity of peritonitis depends on the adequacy of the immune response. Purpose of research was to study the features of cellular arm of immune response in the organism of experimental animals with simulated AGP in the setting of diabetes mellitus (DM). 56 white rats were used in the experiment, which was divided into three groups: main group – 24 animals with simulated AWP in the setting of DM; comparison group – 24 animals with simulated peritonitis. Animals of the main group AGP were modelled by injecting 10 % of filtered fecal suspension into the abdominal cavity of tested rats at a dose of 0,5 ml per 100 g of body weight. Removal of material for histological examination was performed on 1, 3 and 7 days. Cellular immunity was determined by a method based on the interaction of fluorescently labeed monoclonal antibodies with lymphocyte surface antigens. In both animals, all indicators of cellular immunity gradually decreased from day 1 to day 7 of the experiment, but these changes were more significant in animals with DM. With AGP, the cellular immune response imbalance is more significant in animals with DM than in animals with isolated AGP, which is characterized by a marked statistically significant decrease in the level of $CD3^+$ cells, $CD4^+$ cells, $CD16^+$ cells, and a moderate increase in $CD8^+$ cells. The imbalance of cellular immunity deepens depending on the duration of the lesion. The level of $CD3^+$ cells at 1, 3, and 7 day in animals with AGP in the setting of DM was 53,77%, 60,48%, and 62,1 %, respectively, lower than the level in the group with animals with AGP. The prolonged imbalance of cellular immunity indices in experimental animals with simulated AGP in the setting of DM indicates not only the occurrence of secondary immunodeficiency, but also significant depletion of the body's immune forces compared to animals with AGP.

Keywords: *cellular immunity; acute generalized peritonitis; simulated peritonitis; diabetes mellitus.*

1. INTRODUCTION

Acute generalized peritonitis (AGP) is increasingly occurring in patients with concomitant pathology, resulting in changes of the mechanisms of its development, worsening treatment effects and, as a consequence, high mortality [1-2]. According to some authors, in severe forms of widespread purulent peritonitis, the mortality rate reaches 50–80 % [3–5]. Such high mortality is due to the fact that peritonitis occurs on the background of pre-existing pathological changes caused by a comorbid condition, in particular diabetes mellitus (DM), the feature of which is the absence of clear boundaries, the rapid spread of pathological process in abdominal cavity, and complexity. The severity of peritonitis depends on the adequacy of immune response. Correct immune response and sufficient reserves of compensation for the body prevent the spread of inflammatory processes in the abdominal cavity. Immune deficiency causes an adverse course of peritonitis, it is characterized as a secondary acquired immunodeficiency state, which causes complications, sepsis and death of patients [6-7].

DM is known to arise from a defect in immunological tolerance to its own antigens by selective destruction of β-cells of pancreatic islets by $CD8^+$ (cytotoxic) and $CD4^+$ (effector) lymphocytes. Today, the main group of lymphoid cells is considered to be the pool of $CD4^+$ lymphocytes that express the $CD2^+$ + marker molecule – α-chain of IL-2 (IL-2R) receptors. The subpopulation of $CD4^+$ $CD25^+$ is called T-regulatory cells, whilst not all of them performing this function, but only their fractions with high $CD25^+$ expression. Their main role – to control the immune response by regulating the function of T-effector cells (T-helper cells and T-cytotoxic cells) [8]. The participation of T-effector cells through the regulation of autoimmune processes in type 1 DM is confirmed by the factor of association of type 1 DM with IPEX syndrome (Immune dysregulation, Polyendocrinopathy, Enteropathy, and X-linked inheritance) – monogeneous autoimmune syndrome, linked to the X chromosome, the main manifestations of which are immune dysregulation, polyendocrinopathy and enteropathy. The state of the immune system is an important determinant of the progression of inflammatory process at peritonitis in the setting of DM. During cell destruction is formed a cellular immune response, mediated by macrophages, T-lymphocytes, natural killer cells, which exhibit a specific (by direct cytolysis) and nonspecific (through the production of inflammatory mediators) cytotoxic effect [9–11].

The potentiation or summation of pathogenetic effects, one of which is the immune response, is decisive in the development of AWP in the setting of existing DM, remains an unresolved question. Therefore, to date, the role of imbalance of immunological homeostasis in the pathogenesis of peritonitis in combination with diabetes mellitus remains unclear and has not been fully studied.

Purpose of research was to study the features of the cellular arm of immune response in the organism of experimental animals with simulated AWP in the setting of DM.

2. MATERIALS AND METHODS

Experiment protocol.

56 white rats were used in the experiment, which was divided into three groups: main group – 24 animals with simulated AWP in the setting of DM; comparison group – 24 animals with simulated peritonitis; control group consisted of 8 intact animal, which was kept in standard conditions of vivarium. All compared groups of animals were representative by weight, sex and age.

Experimental DM was reproduced by intraperitoneal administration of streptozotocin on an empty stomach at a dose of 60 mg/kg (firm "Sigmal", which was dissolved in a buffer natrium and sodium citrate pH 4.5). Studies of glucose content were carried out by the glucose-oxidant method at 9:00 under conditions of free access of experimental animals to food and water during the night period of time. Insulin (0-2 IU subcutaneously two to five times per week) was administered to rats throughout the observation period.

After 2 weeks from the use of streptozotocin in rats from venous blood, which was obtained from the tail vein, was determined the glucose content and in subsequent studies, only those rats were observed in which glucose content was more than 300 mg/l. In animals of the control group were administered subcutaneously sterile 0.9% sodium chloride solution instead of streptozotocin [11].

The influence of DM on the course of AWP was studied on the model proposed by Lazarenko V.A. et al. [12]. This model is close to a similar process in humans in terms of etiological factors, clinical manifestations and phase of the course. On the 14th day after the administration of streptozotocin, animals of the main group were injected with 10% of filtered fecal suspension into the abdominal cavity of studied rats at a dose of 0,5 ml per 100 g of body weight. Rats of the comparative group received an only subcutaneous injection of fecal suspension. The fecal suspension was obtained by mixing isotonic solution and faeces from the cecum of 2–3 intact animals, then filtered twice through a double layer of gauze. The received suspension was injected into the intact rats in a puncture manner no later than 20 min after preparation. In order to avoid damage to the internal organs when the fecal suspension was introduced into the abdominal cavity, the animals were kept upright, with a caudal end up. Using the method of puncture of the ventral wall in the center of the midline of abdomen, directing the end of needle alternately into the right and left hypochondrium, right and left iliac areas, was introduced the same amount of fecal suspension.

The terms of observation were 1, 3, and 7 day. This experimental study was conducted in accordance with the general rules and regulations of the European Convention on the Protection of Vertebrate Animals, which are used for research and other scientific purposes (Strasbourg, 1986).

Study of cellular immunity.

Indicators of cellular immunity were determined by a method that was based on the interaction of monoclonal antibodies (MCAb), marked with a fluorescent label, with lymphocyte surface antigens. Determination of subpopulations of T- and B-lymphocytes was determined using Rat ELISA Kits ("NeoScientific" and "MyBioSource", USA).

20 μl of antibodies ($CD3^+$/$CD4^+$/$CD8^+$, $CD16^+$) and ethylenediaminetetraacetic acid were introduced into each bulb without touching the bulb wall. The samples were stirred in a vortex and incubated in the dark for 15-30 min. at room temperature [13]. The recommended number of leukocytes – 3,5-9,4 g/l. For erythrocyte lysis, 500 μl of lysis solution was introduced into each bulb, stirred in a vortex, and incubated for 10-15 min. at room temperature. 500 μl of buffer solution was added to the bulbs. The samples were analyzed on an Epics-XL flow cytometer manufactured by Beckman Coulter (USA).

Statistical analysis.

Statistical processing of the received data was performed on a personal computer using standard software packages of Microsoft Excel and with the help of the computer program Statistica for Windows version 6.0 (Stat Soft inc., USA). The results were presented as mean values (M) ± the error of the mean (m) and were tested by one-way ANOVA, followed by Fisher's least significant difference procedure as a post-hoc test. A level of $P<0.05$ was considered significant.

3. RESULTS

As shown by our studies, the level of total T-lymphocytes (CD3 + cells) in the group of animals with simulated AWP decreased 1,9 times compared with intact animals as early as 1 day after AWP modelling. A similar tendency was observed in the group of animals with AWP in the setting of DM. The level of $CD3^+$ cells at 1st day after the simulation of experiment decreased 4,1 times compared with intact animals, which is 53.78% statistically significantly less compared to the group of animals with simulated AWP. On 3rd day after modeling of peritonitis, the level of $CD3^+$ cells in AWP group and the AWP+DM group decreased by 49,18% and 79,90%, respectively, compared to control.

Table 1. Indicators of cellular immunity in rats with simulated AWP (m ± M).

Terms of observation	CD3+, $\times10^9$ in 1 L	CD4+, $\times10^9$ in 1 L	CD8+, $\times10^9$ in 1 L	CD16+, $\times10^9$ in 1 L
Intact group	5,82±0,22	1,35±0,14	2,23±0,19	3,81±0,20
1 day (n=8)	3,05±0,23	0,86±0,12	1,58±0,24	2,57±0,13
3 days (n=8)	2,96±0,31	0,83±0,11	1,61±0,28	2,47±0,17
7 days (n=8)	2,75±0,13*\#	0,79±0,13	1,65±0,23*	2,36±0,24*\#

Note: * - reliability of difference of indicators in comparison with 1st day; # - reliability of difference of indicators in comparison with 3rd day.

A more significant reduction of $CD3^+$ cells was obtained on 7th day of the experiment. During this period, the level of $CD3^+$

cells in AWP+DM group was 62,50% statistically significantly lower than the animal group with simulated AWP.

T-helper cells ($CD4^+$ cells) and T-suppressors ($CD8^+$ cells) are components of the common part of T-cell system. Therefore, it is naturally that we observed the same dynamics of regulatory T-lymphocytes as in the animal group with model AWP and in the animal group with AWP in the setting of DM. However, this decrease in indices was more significant in the AWP + DM group during the whole study. The level of regulatory $CD4^+$ cells in animals with AWP at 1st day was 1,5 times lower than that of the intact animal group. In animals with AWP in the setting of DM, this index also decreased and was 3,6 times lower than the control. Similar dynamics were observed on 3rd and 7th day. In animals with simulated AWP, $CD4^+$ cell level decreased 1,6 times at 3rd day, and 1.7 times at 7th day compared with the control. With regard to the group of animals with AWP in the setting of DM, the decrease in the level of $CD4^+$ cells was more significant. On 3rd day this indicator decreased 4,0 times, and on 7th day – by 4,5 times compared to the control. Determining the $CD4^+$ cell fraction at 1, 3, and 7 days in animals with AWP in the setting of DM, we observed a statistically significant decrease of 55,82%, 59,04%, and 62,03%, respectively, compared with the AWP group. With respect to $CD8^+$ cells, their level increased slightly depending on the observation period compared to the level of this indicator in intact animals. In animals with simulated AWP, the level of $CD8^+$ cells at 1st day was 29,15% lower compared to intact animals, and in animals with AWP in the setting of DM – 58,37%. At 3rd day, the level of these regulatory T-lymphocytes in the AWP group was 27,81% lower than the control, and at 7th day – 26,01%. In the group with simulated AWP in the setting of DM at 3rd and 7th day there was a significant decrease in this indicator. On 3rd day, $CD8^+$ cell level was 56,23% lower than intact animal level, and at 7th day – 54,94%. We also determined a statistically significant decrease in the $CD8^+$ cell fraction at 1, 3, and 7 day in animals of AWP+DM group by 38,61%, 36,65%, and 36.37%, respectively, compared with AWP group.

Table 2. Indicators of cellular immunity in rats with simulated AWP in the setting of DM (m±M).

Terms of observation	CD3+, $\times10^9$ in 1 L	CD4+, $\times10^9$ in 1 L	CD8+, $\times10^9$ in 1 L	CD16+, $\times10^9$ in 1 L
Intact group	5,82±0,22	1,35±0,14	2,23±0,19	3,81±0,20
1 day (n=8)	1,41±0,20	0,38±0,16	0,97±0,18	1,28±0,19
3 days (n=8)	1,17±0,22*	0,34±0,15	1,02±0,17	1,20±0,23*
7 days (n=8)	1,02±0,18*\#	0,30±0,21*	1,05±0,13*	1,16±0,26*\#

Note: * - reliability of difference of indicators in comparison with 1st day; # - reliability of difference of indicators in comparison with 3rd day.

With regard to natural killer cells ($CD16^+$ cells), their levels also decreased in all study periods in the two comparison groups. The level of $CD16^+$ cells in AWP+DM group at 1st day of experiment was 50,20% statistically significantly lower than AWP group, at 3rd day – 51,42%, and on 7th day – 55,90%. The presented data showed that the level of natural killer cells is significantly reduced in the background of severe intoxication with simulated AWP in setting of DM.

Peritonitis occurs on the background of immunodeficiency, and concomitant pathology, in particular, diabetes mellitus causes more pronounced immune deficiency. Diabetes mellitus occurs when there is a defect in immunological tolerance to its own antigens and selective destruction of β-cells of pancreatic islets by $CD8^+$ (cytotoxic) and $CD4^+$ (effector) lymphocytes. The summation of immunological changes in the combined pathology exacerbates the course of the main disease, which we have been able to prove. Changes in the immune system are associated with neuroregulatory disorders, activation of chemoattractants and involvement in the process of inflammatory cells, impairment of local and systemic metabolism and hemodynamics [2, 11, 14, 15]. There is no doubt that in peritonitis with DM, immune and metabolic processes run in parallel and underlie common mechanisms associated with the imbalance between the production of pro-inflammatory and anti-inflammatory cytokines, as well as with corresponding changes in the activity of neutrophils and macrophages as effector cells [7, 8]. A feature of the status of cellular level of immunity in our experiment is the decrease in the percentage and absolute number of T-lymphocytes, which indicates an increase in cytotoxic and antigen-presenting effect, because these cells are inherent in this function. In addition, this population may exhibit regulatory activity.

It was shown that the concentration of total T-lymphocytes ($CD3^+$ - cells) a day after the AWP simulation was 1,9 times lower than the control. A similar tendency was observed in the group of animals with AWP in the setting of DM. The level of $CD3^+$ cells at 1st day after the simulation of experiment decreased 4,1 times compared with intact animals, which is 53,78% statistically significantly less compared to the group of animals with simulated AWP. Such abnormalities are associated with marked pain syndrome and induced stress, as a result of the release of catabolic hormones that block the migration of immunocompetent cells from thymus [3, 4] and the redistribution of circulating lymphocytes from the vascular bed into damaged tissues. On 3rd day, a further decrease by 49,18% and 79,90% were observed, respectively, compared with control of the amount of $CD3^+$ cells in animals with simulated AWP and AWP in the setting of DM. A more significant reduction in $CD3^+$ cells was obtained on 7th day of the experiment. During this period, the level of $CD3^+$ cells in AWP+DM group was 62,50% statistically significantly lower than the animal group with the simulated AWP. The development of any systemic pathology, including AWP for the body, is a powerful stress-inducing factor. T-lymphocytes, both the general population and its subpopulations, express on the membrane a significant number of receptors for glucocorticoids, which causes cell death in apoptosis at elevated concentrations of these hormones in the body. The mechanism of such death of T-lymphocytes is realized at least 40-48 hours after the development of stress. Therefore, the obtained significant decrease in the content of T-lymphocytes at this time programmed phenomenon of cell death in apoptosis.

Subpopulations of regulatory T-lymphocytes, namely: T-helper cells ($CD4^+$ - cells) and T-suppressors ($CD8^+$ - cells) are constituents of the total fraction of the T-cell system. Therefore, it is quite natural that there is the same dynamics of general T-lymphocytes as AWP develops in the setting of DM. However, the degree of change in each subpopulation is clearly different. Determining the fraction of $CD4^+$ cells at 1, 3, and 7 days in AWP + DM animal group, we observed a statistically significant decrease of 55,82%, 59,04%, and 62,03%, respectively, compared

with AWP group. Such dynamics of CD4 level is an unfavorable prognosis, a sign of insufficient activation of protective regulation of specific immunity. Such redistribution of subpopulations with a predominant content of T-suppressors/cytotoxic lymphocytes indicates a pronounced dysregulatory state in AWP of those cells, which largely determine the level of realization of immunoinflammatory response. With regard to $CD8^+$ cells, their level increased somewhat depending on the observation period. We determined a statistically significant decrease in $CD8^+$ cell fraction at 1, 3, and 7 days in AWP+DM animal group by 38,61%, 36,65%, and 36,37%, respectively, compared with the simulated AWP animal group.

In recent years, attention has been focused on cells that implement effector protective function of the body on natural killer cells ($CD16^+$ cells). The presented data showed that, against the background of significant intoxication with simulated AWP, the content of these cells decreased by 32,55% - on 1st day, by 35,17% - on 3rd day, and by 38,06% - on 7th day compared to the control that was due to the violation of water, electrolyte, carbohydrate and vitamin metabolism.

However, expressed intoxication leads to violation of protein metabolism and liver function – accumulate intermediate metabolites of metabolism. In such circumstances, the immune system shows its failure, which can be a consequence of an imbalance of neuroimmune vector. It is likely that immunological disorders and chronic inflammation that occur with DM may worsen the course of peritonitis and its complications. Further studies to determine the features of the immune status in patients with AWP in the setting of DM may be the basis for substantiating treatment options for the investigated comorbid condition.

4. CONCLUSIONS

With AWP, the cellular immune response imbalance is more significant in animals with DM than in animals with isolated AWP, which is characterized by a marked statistically significant decrease in the level of $CD3^+$ cells, $CD4^+$ cells, $CD16^+$ cells, and a moderate increase in $CD8^+$ cells. The imbalance of cellular immunity deepens depending on the duration of the lesion. The level of $CD3^+$ cells at 1, 3, and 7 day in animals with AWP in the setting of DM was 53,77%, 60,48%, and 62,1%, respectively, lower than the level in the group with animals with AWP. The prolonged imbalance of cellular immunity indices in experimental animals with simulated AWP in the setting of DM indicates not only the occurrence of secondary immunodeficiency, but also significant depletion of the body's immune forces compared to animals with AWP.

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