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Effect of 1,2-Dimethylhydrazine on Spleen Immunomorphology

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***Abstract* — The article presents the main morphological and immunohistochemical changes of the spleen in rats in 1 and 4 months after the course injection of 1,2-dimethylhydrazine. The increase in the number of $CD68^+$-cells in the red pulp and S100+-cells in the germinal centers of lymphoid nodules was registered 1 month after injection in the spleen. The number of $CD79\alpha^+$ and $CD45RO^+$ cells in white pulp structures, on the other hand, decreases. The area of lymphoid nodules and their germinal centers, the width of the marginal zone and periarterial lymphoid couplings are reduced. The total number of secondary lymphoid nodules increases. Four months after the end of the course of carcinogen injections, there is a significant increase in the number of $CD68^+$-cells in the red spleen pulp. Changes in the number of $CD68^+$ and $S100^+$ cells in germination centers are unreliable. The number of $CD79\alpha^+$ and $CD45RO^+$ cells is significantly reduced. Changes in the morphological phenotype of the spleen at this time consist in a more pronounced reduction in the area of the lymphoid nodules with their germinal centers, the width of the periarterial lymphoid couplings and the marginal zone, as well as an increase in the number of secondary lymphoid nodules.**

Keywords — spleen, carcinogenesis, 1,2-dimethylhydrazine, white pulp, red pulp.

I. INTRODUCTION

The global problem of recent decades remains the high prevalence of morbidity and mortality from malignant neoplasms. According to the International Agency for Research on Cancer (IARC/IARS, Lyon), the number of cancer cases worldwide in 2018 was a record high of more than 18 million. According to the preliminary forecast, this figure is expected to increase to 29.5 million people by 2040. And the number of deaths from malignant tumors will increase from 9.5 million to 16.3 million. There are many reasons that lead to cell malignization. These include environmental pollution, age, viruses, acute and chronic stress as well as hereditary component, hormonal shifts and imbalances in the immune system. Food with chemical dyes and preservatives, as well as carcinogens of chemical, physical or biological nature can also lead to tumor growth in the body [4]. The only weapon against carcinogenesis in the body is the immune system, with which the growing tumor has a complex and yet unexplored relationship. This problem is studied by a relatively young science – oncoimmunology [2].

1,2-dimethylhydrazine dihydrochloride (DMH) is a derivative of hydrazine, the carcinogenicity of which has been known for more than 40 years. According to the classification of carcinogens developed by IARC, DMH is a group of substances that are probably carcinogenic to humans. It is used to study carcinogenic properties, namely, to model colon cancer in laboratory animals [5, 12]. This model has been sufficiently studied; unlike cell cultures, it allows to reproduce tumor angiogenesis and metastasis [3]. There are reports that vascular tumors may occur even in small doses during peroral administration of DMH [7]. It is also known that a single subcutaneous injection of this carcinogen leads to cytotoxic affection of enterocytes, which is manifested by changes in apoptosis indexes and cell proliferation [11]. Introduction of 1,2-dimethylhydrazine to rats in the course dose of 40 mg/kg leads to toxic damage to the walls of the large intestine [16]. It

is known that this carcinogen leads to the development of the excidental involution of the thymus [17], as well as to morphological changes in the organs of the endocrine system [18]. There is evidence in the available literature that DMH injections result in marked disturbances in the morphological state of all structural components of the spleen [8]. In view of these findings, a more in-depth study of spleen morphology using general histological and immunohistochemical methods is highly relevant.

II. MATERIAL AND RESEARCH METHODS

A. Research material

The studies were performed on 70 white non-linear male rats. The animals were kept in a vivarium and cared for according to the norms and rules of work with laboratory animals according to the European Convention for the Protection of Vertebrate Animals and the current GOST 33216-2014 [14]. The work was carried out on the basis of the Faculty of Medicine of the Federal State Budgetary Educational Institution "Chuvash State University named after I.N. Ulyanov".

To create an experimental model of colon cancer as a carcinogen was used 1,2-dimethylhydrazine dihydrochloride, manufactured by Acros organics (Belgium). The animals were divided into two groups: the first – intact (n=30); the second – rats with intraperitoneal administration of 1.2-dimethygidrazine 1 time per week in a dose of 20 mg/kg for one month according to the R.F. model. Jacoby (n=40) [6]. In order to exclude the influence of the stress factor from carcinogen injection on the results of the study, an intraperitoneal sodium chloride isotonic solution was injected into the control animals (n=10) in a dose of 0.5 ml 1 time per week during one month. It was found that injections of isotonic solution in control animals in comparison with intact rats had no effect on the studied structures in the terms used in this study. Withdrawal from the experiment was carried out 1 and 4 months after the end of the course of injections by decapitation, i.e. at the age of 3 and 6 months, respectively. In this study, only rats with a pathomorphologically confirmed colon adenocarcinoma were used to study the spleen.

B. Research methods

1. Hematoxylin and eosin staining for spleen morphology and morphometric measurements. The spleen tissue was fixed in 10% neutral buffered formaldehyde for 24 hours, washed with running water, then performed standard wiring on Leica ASP 200 tissue histoprocessor. After the wiring, pieces of spleen tissue were poured into paraffin and paraffin blocks were prepared. Wax spleen slices of 4 microns thickness were applied to negatively charged glasses of Mentzel Glasses super frost and stained with hematoxylin and eosin according to the standard method.

2. Immunohistochemical method. The study was carried out in accordance with the standard protocols. The material for the study was fixed with 10% neutral buffered formaldehyde for 24 hours, than poured into paraffin and prepared cuttings. Fabric cuts of 4 μm thick were applied to high-adhesive glass and dried at room temperature for 24 hours. The restoration of antigenic activity was carried out in a citrate buffer at pH 6.0 in an autoclave at 96 °C for 30 min, followed by cooling at room temperature for 90 min. Painting was performed manually using Leica ChromoPlex™ 1 Dual Detection for BOND imaging systems. Sensitivity and specificity of the reaction was controlled by nonimmunized serums. Monoclonal (MCA) antibodies to CD68 (phagocytic macrophages), CD79α (B-lymphocytes), CD45RO (T-memory cells) and polyclonal antibody (PCAT) to S100 (dendritic cells) were used in this study.

2. Computer morphometry. The digital images of the micro preparations were obtained with the use of the archiving system based on the MIKROMED 3 LUM microscope using a digital camera and a personal computer with a set of applications. Microphotographs for morphometric measurements were obtained at magnifications of x100 and x400. Quantitative morphometric measurements were made using the Micro-Analysis license program. The area of membrane and cytoplasmic immunohistochemical reaction was estimated by the method of automatic isolation and calculation of the area of the color spectrum of interest (colored DAB) in relation to the image area. The numerical values of the area of the positive immunohistochemical reaction were then converted into a percentage of the total image area. For each cut, measurements are made in at least 10 fields of interest.

4. Statistical processing of digital data was carried out with the help of the Microsoft Office 2003 license package (Word and Excel) and the G-Stat software, the reliability of which was determined by the Student's criterion (t). The following indicators are provided in the paper: M – average arithmetic value; m – average error of average arithmetic value. The differences at $p<0.05$ [13] were considered reliable.

III. RESULTS AND DISCUSSION

In histological treatment of the spleen of intact rats at the age of 3 months has a typical and, as noted by many scientists, the most complicated structure among peripheral immune organs [1]. Outside the spleen is covered with a well-defined connective tissue capsule, from which trabeculae depart to the parenchyma layer. The organ parenchyma is represented by red and white pulp. The red pulp is 75–85 % of the organ mass, including pulp rods and sinusoids. White pulp is represented by lymphoid nodules and periarterial lymphoid couplings (PALM). Among lymphoid nodules there are primary nodules without germinative centers and secondary nodules with breeding centers, which appeared in them after antigenic stimulation. 1–3 lymphoid nodules are visualized in one field of view, with the ratio between secondary and primary lymphoid nodules being 2.6:1. Lymph nodules have a clear zonal structure, each of which defines an eccentrically located central artery, periarterial T-dependent zone, germinal center (B-dependent zone), mantle and marginal zones.

There are no significant differences from spleen of 3-month-old animals on histological spleen slices of intact animals at the age of 6 months. There is a red and white pulp, the boundaries between them are still clear, the number of

lymphoid nodules does not change significantly, but the ratio of lymphoid nodules with and without breeding centers is 1.3:1.

Immunohistochemical studies revealed that the largest number of $CD68^+$ cells is found in the red spleen pulp. The content of $CD68^+$-cells in white pulp in the control group animals is relatively low. We identified two populations of $CD68^+$-cells – small (up to 50 μm^2 in area) and large (from 50 to 200 μm^2) cells. It is known from the literature that monocytes can be referred to small cells, and macrophages can be referred to large cells [20]. Large $CD68^+$-white spleen pulp cells may belong to intrafollicular dendritic macrophages formed from monocytes and participating in the presentation of B-lymphocytes [20]. The spleen is dominated by small cells located mainly in the red pulp. In the morphofunctional areas of the white pulp, except for small cells, there are single large cellular elements.

The main pool of S100+-cells is distributed in the white pulp – the germinal centers of lymphoid nodules and mantle zone (Fig. 1). There is evidence in the literature that DC spleen actively participates in antitumor immune monitoring. However, there are conflicting data regarding the functional orientation of these cells in the antitumor immune response. Some authors believe that spleen dendritic cells have a high antitumor potential and are able to inhibit tumor cell growth, whereas thymus dendritic cells do not possess such activity [15]. Other authors believe that spleen dendritic cells, on the other hand, may contribute to the tumor's slippage from immune supervision [10].

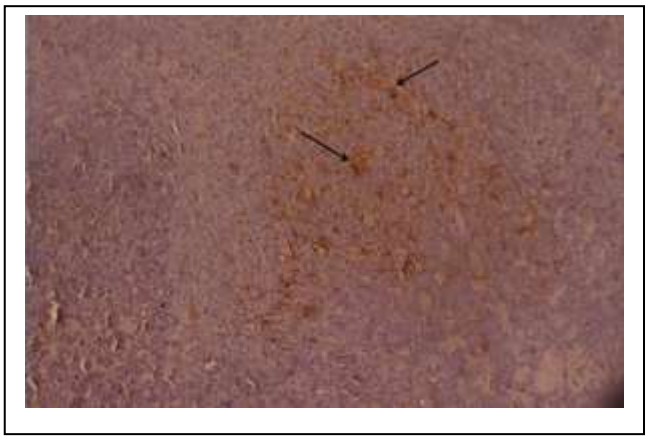

Fig. 1. S100+-cells accumulation in the germinal centres of lymphoid nodules (painted brown) of intact rats. Age 3 months. Immunohistochemical reaction of cells to protein S100. Magnification 400.

The expression of CD45RO is defined in the T-dependent areas of white pulp and in a small amount in the red pulp. $CD79\alpha^+$-cells are defined in the B-dependent and marginal zones, as well as in the spleen rods of red pulp. B-lymphocytes of the spleen synthesize a number of antigen-specific immunoglobulins and other nonspecific biologically active substances, including serum tetrapeptide tetrapeptide taftsin. It is a universal stimulator of phagocytosis and enhances its and other functional properties of macrophages and polymorphonuclear leukocytes even in small doses [19].

One month after 1,2-dimethylhydrazine administration, the white spleen pulp retains its zonal structure, but the contours of the lymphoid nodules and polymorphonuclear leukocytes become less distinct. The number of secondary lymphoid nodules does not change reliably, the ratio between primary and secondary nodules decreases to 1.7:1. It was found that the introduced carcinogen leads to a relative increase in the number of small and medium (from 300 to 500 microns in diameter) lymphoid nodules. At the same time, the area of lymphoid nodules is reduced (by 14 %) and their diameter – by 19%. The area of the germination centers of the secondary nodules decreases by 21 %, the diameter – by 12 % (Table 1)

TABLE I. MORPHOMETRIC PARAMETERS OF WHITE SPLEEN PULP

	Age 3 months		**Age 6 months**	
	Intact animals	**Carcinogen administration (after 1 month)**	**Intact animals**	**Carcinogen administration (after 4 months)**
Square of lymphoid nodules, $\times 10^3$ μm	470,21±20,7	402,7±14**	530,3±31,2	394,21± 30,1***
Diameter of lymphoid nodules (DLF), μm	620,85±23,5	502,33± 18,4***	561,87±22,9	437,71± 14,3***
Area of germination centres, ×103 μm	49,31±3,36	38,9±1.98**	60,76±3,5	49,45±4,1*
Diameter of germination centres (DHz), μm	218,53±8,5	191,59±8,3*	202,025±9,2	171,78± 5,9**
PALM width (LPM), microns	118,31±4,8	125,06±4,8	119,33±3,3	111,11±3,9
Width of marginal zones, microns	121,36±6,02	118,35±2,2	122,85±2,7	109,73± 2,1***

* – $p < 0,05$; ** – $p < 0,01$; *** – $p < 0,001$ compared to the same indicators in intact animals.

More significant morphometric changes are observed 4 months after the end of the course of carcinogen injection. Lymph nodules lose their proper shape, the contours between the zones become more blurred and erased. The number of secondary lymphoid nodules increases significantly, but the ratio between them and the primary nodules decreases to only 1.5:1, which may indicate a decrease in antigenic stimulation. The area of lymphoid nodules is decreasing, amounting to 25 %, which is more significant compared to the changes in the previous term. The area of the germination centers of secondary follicles is reduced by 18%, and their diameter – by 15 %. The width of the marginal zone in animals decreases significantly by 11 % in 4 months after exposure to carcinogen, and the width of periarterial lymphoid couplings does not change significantly (Table 1).

One month after the end of the course of injections of 1.2-dimethylhydrase, a significant increase in the number of CD68-positive cells in the red spleen pulp by 72 % was

detected. Four months after exposure to carcinogen, the number of macrophages increases further, to 82 % of the increase compared to intact animals of the same age (Table 2). Carcinogen did not lead to significant changes in the number of white pulp cells under study. It was revealed that rats had a significant increase in the number of S100-positive cells in the germinal centers (Fig. 2) 1 month after the administration of carcinogen, and rats had an unreliable change in the number of these cells in the spleen 4 months after exposure (Table 2).

Fig. 2. Accumulation of S100+ cells in the germinal centres of lymphoid nodules (coloured brown) in rats 1 month after administration of carcinogen. Age 3 months. Immunohistochemical reaction of cells to protein S100. Magnification 400.

In addition, our studies have shown that the number of CD79α+ cells decreases during carcinogenesis in B-dependent zones of lymphoid nodules. One month after exposure to carcinogen, the number of CD79-α-positive cells decreases by 31 %, and 4 months after the end of the course by 35 % (Table 2) The expression of CD45RO in white spleen pulp decreases in animals both 1 and 4 months after administration of 1.2- dimethylhydrase. However, the most pronounced changes are observed in animals 4 months after exposure to carcinogen (Table 2).

TABLE II. IMMUNOHISTOCHEMICAL REVISION OF SPLEEN CELLS AFTER INJECTIONS OF 1,2-DIMETHYLHYDRAZINE, %

	Age 3 months		**Age 6 months**	
	Intact animals	**Carcinogen administration (after 1 month)**	**Intact animals**	**Carcinogen administration (after 4 months)**
CD68+ red pulp	18.47±1.26	31.96±4.9*	22.69±5.1	41.51±2***
CD68+ white pulp	31.63±1.9	30.2±4.6	37.75±1.5	38.13±1.8*
S100+	21.82±1.6	44.57±3*	32.41±2.42	31.72±1.56
CD79α+	49.91±2.5	32.48±3.5**	45.37±3.5	29.31±3.6**
CD45RO	42.39±2.7	35.24±2*	38.07±2.3	32.36±2**

* – $p < 0,05$; ** – $p < 0,01$; *** – $p < 0,001$ compared to the same indicators in intact animals.

IV. CONCLUSION

Thus, the data obtained indicate that the injected 1,2-dimethylhydrazine has a negative effect on the morphology of the spleen. The administration of this carcinogen in a dose equivalent to the development of colon adenocarcinoma has led to marked disturbances in the morphological state of all structural components of the spleen with varying degrees of severity in the study groups. One month after the end of the course of carcinogen injections it manifested itself in destructive-dystrophic changes of white pulp with significant changes in its cellular ratio. There is a reduction in the area and diameter of the spleen lymph nodes, a reduction in the diameter of the germinal centers, the width of the periarterial lymphoid couplings and the marginal zone. Such changes characterize the stress of humoral and cellular links of immune supervision, which, in turn, leads to a lack of antitumor immune response. The effects of 1,2-dimethylhydrazine administration were more evident in animals 4 months after injection. This was characterized not only by a more pronounced hypoplasia of the white spleen pulp, but also by even greater changes in the cell population of the organ. Populations of S100, $CD75\alpha^+$, $CD45RO^+$ and $CD68^+$ cells in the spleen were highly vulnerable to 1,2-dimethylhydrazine. More pronounced changes in the population of these cells were observed in animals 4 months after the action of the carcinogen. The decrease in the number of CD68+-cells in the white pulp and, on the contrary, their increase in the red pulp, is explained by the possible migration of macrophages and dendritic cells from the blood [9]. Significant morphometric changes in the white spleen pulp and its cell count in animals 4 months after exposure to carcinogen may be associated with age-related changes in the spleen and the beginning of organ hypoplasia. The changes we found may eventually lead to the disruption of interaction between CD68+-cells and S100+-cells with antigens. This will lead to the disruption of differentiation of B-lymphocytes and T-lymphocytes and, consequently, to a decrease in the intensity of humoral and cellular links of anticancer immune response.

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