IMMUNOMODULATOR IMUNOFAN AFFECTS CELL PROFILE OF MORPHOFUNCTIONAL ZONES OF RAT THYMUS AND DELAYS ITS AGE-RELATED INVOLUTION

Bobrysheva IV ✉

Department of Histology, Cytology and Embryology, Faculty of Stomatology,
Lugansk State Medical University, Lugansk, Ukraine

The immunomodulatory agent Imunofan (Bionox, Russia) is widely used in clinical practice. It affects the immune and endocrine systems and enhances cell-mediated and humoral immunity. The aim of this study was to investigate the cell profile (lymphoblasts, small, medium and large lymphocytes, macrophages, mitotic cells and damaged cells) in the subcapsular and inner zones of the thymic cortex and thymic medulla of random-bred male albino rats with conspicuous age-related changes after stimulating their immune system with Imunofan. The animals in the experimental group (n = 30) were administered to intramuscular injections of the drug (0.7 mg/kg) on the 1st, 3rd, 5th, 7th and 9th days of the experiment; the controls (n = 30) were administered to the equivalent amount of normal saline on the same days. Rats were decapitated on the 1st, 7th, 15th, 30th and 60th days after the final injection. Thymic sections were studied using Olympus CX-41 microscope, Olympus SP 500UZ camera (Olympus, Japan) and Morpholog software (Ukraine). Thymic morphology was similar in the experimental and control groups; however, cell profiles were different. On the 7th, 15th and 30th days, lymphoid cells and macrophages prevailed over damaged cells, the number of which decreased ($p < 0.05$). Similar statistically significant trends were found in the inner zone of the thymic cortex. The number of medium lymphocytes was statistically higher on the 7th, 15th and 30th days of the observation, while the number of small lymphocytes was also higher on the 60th day of the observation. The number of damaged cells was significantly lower on the 15th and 30th days ($p < 0.05$). The obtained results indicate conspicuous thymic response in rats with conspicous age-related changes to Imunofan administration, and partial temporary delay of age-related thymic involution.

Keywords: thymus, age-related involution, immune stimulation, immunomodulator, Imunofan, albino rats

✉ **Correspondence should be addressed:** Inessa Bobrysheva
ul. 15-ya Liniya, d.18, kv. 8, Lugansk, Ukraine, 91016; inessa_lug@mail.ru

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ИММУНОМОДУЛЯТОР «ИМУНОФАН» ВЛИЯЕТ НА КЛЕТОЧНЫЙ СОСТАВ МОРФОФУНКЦИОНАЛЬНЫХ ЗОН ТИМУСА КРЫС И ЗАМЕДЛЯЕТ ЕГО ВОЗРАСТНУЮ ИНВОЛЮЦИЮ

И. В. Бобрышева ✉

Кафедра гистологии, цитологии и эмбриологии, стоматологический факультет,
Луганский государственный медицинский университет, Луганск, Украина

В клинической практике применяется иммуномодулятор «Имунофан» («Бионокс», Россия), воздействующий на иммунную и эндокринную системы и усиливающий клеточный и гуморальный иммунитет. Целью исследования являлось изучение содержания лимфобластов, малых, средних и больших лимфоцитов, макрофагов, митотически делящихся и деструктивно измененных клеток в субкапсулярной и внутренней зонах коркового вещества и мозговом веществе паренхимы тимуса беспородных белых крыс-самцов периода выраженных старческих изменений при иммуностимуляции «Имунофаном». Животным опытной группы (n = 30) вводили препарат внутримышечно из расчета 0,7 мг/кг в 1, 3, 5, 7 и 9-е сутки эксперимента, а животным контрольной группы (n = 30) — эквивалентный объем физиологического раствора в те же сроки. Декапитацию осуществляли на 1, 7, 15, 30 и 60-е сутки после последней инъекции. Срезы изучали с помощью микроскопа Olympus CX-41, фотоаппарата Olympus SP 500UZ (Olympus, Япония) и программного пакета Morpholog (Украина). Морфологические особенности органа в опытной и контрольной группах были схожими, но клеточный состав зон различался. В субкапсулярной зоне на 7, 15 и 30-е сутки было больше клеток лимфоидного ряда и макрофагов при одновременном снижении числа клеток с признаками деструкции ($p < 0,05$). Аналогичные статистически значимые закономерности были выявлены для внутренней зоны. В мозговом веществе содержание средних лимфоцитов было достоверно выше на 7, 15 и 30-е сутки наблюдения, а малых лимфоцитов — также и на 60-е сутки. Количество деструктивно измененных клеток значительно уменьшилось на 15 и 30-е сутки ($p < 0,05$). Полученные результаты свидетельствуют о заметной реактивности тимуса крыс периода выраженных старческих изменений на введение «Имунофана» и временном частичном замедлении его возрастной инволюции.

Ключевые слова: тимус, возрастная инволюция, иммуностимуляция, иммуномодулятор, имунофан, белые крысы

✉ **Для корреспонденции:** Бобрышева Инесса Венедиктовна
91016, Украина, г. Луганск, ул. 15 линия, д. 18, кв. 8; inessa_lug@mail.ru

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Thymus is a primary organ of the immune system. It largely contributes to the intensity of immune response and maintains immune homeostasis. Morphological changes in the thymus triggered by a variety of factors, including administration of immunotropic drugs, are accompanied by changes in the cellular microenvironment and cytoarchitecture [1–7].

The immunomodulatory agent Imunofan is widely used in clinical practice. It positively affects the immune and endocrine systems and enhances cell-mediated and humoral immunity [8]. It is an immunoregulatory hexapeptide (arginil-alfa-aspartil-lysil-valil-tyrosil-arginine) synthesized from a thymopoietin fragment that contains amino acid residues of its active site. Pharmacological effects of Imunofan include fine-tuning of the immune system and elimination of oxidative/antioxidative imbalance. Drug action starts 2–3-hours after the injection and lasts up to 4 months. The time course of the drug action can be divided into three phases. During the first phase (2–3 days after the injection), a detoxifying effect of Imunofan is observed: the drug normalizes lipid peroxidation and inhibits breakdown of cell membrane phospholipids and arachidonic acid synthesis. During the second phase that lasts for 7–10 days, phagocytic activity increases followed by the death of intracellular bacteria and viruses. During the final phase that lasts up to 4 months, impaired functions of cell-mediated and humoral immunity are restored.

Effects of Imunofan on different animal and human body systems have been studied widely [1, 3, 9–12]. However, little attention has been paid to the changes in the thymic cytoarchitecture induced by Imunofan in subjects of various age. The aim of this study was to investigate the cell profile of thymic parenchyma in aging rats after stimulating it with Imunofan.

METHODS

The study was conducted in 60 random-bred male albino rats (age of 20 months, weight of 300–330 g). The animals were housed at 20–25 °C, humidity below 50 %, 12 h light (from 8:00 to 20:00) in standard plastic cages, 6 rats per cage, with free access to food and water [13]. According to daily observations, all animals were active and healthy.

The animals were divided into two groups, of 30 rats each. The experimental group received 0.7 mg/kg IM Imunofan (Bionox, Russia; Registration Certificate UA/0318/01/01) once a day on days 1, 3, 5, 7, and 9 of the experiment (the route of administration was chosen according to manufacturer's recommendations; human dosage was converted to animal dosage). The controls were administered to sodium chloride 0.9 % IM in the same amount and on the same days. In each group, rats were sacrificed in sixes 1, 7, 15, 30 and 60 days after the final injection of the drug or sodium chloride solution (the animals were anesthetized with ether and decapitated).

The object of our study was the thymus. Sampling, fixation and paraffin block preparation were performed according to standard techniques for lymphoid tissue processing [14]. To study thymic structural components, 4–6 μm thick paraffin sections were stained with hematoxylin and eosin; for cell identification, azure II and eosin were used. Histological analysis was performed on Olympus CX-41 microscope, using Olympus SP 500UZ digital camera (Olympus, Japan) and Morpholog software (Ukraine) [15]. Microphotographs were taken in various magnification modes, using objective lenses PlanC N x10/0.25∞/–/FN22, PlanC N x40/0.65 ∞/0.17/FN22, PlanC N x60/0.80∞/0.17/FN22, with zoom 132 and 142. From each thymus, six sections were obtained; six fields of view were analyzed in each case, which is sufficient for obtaining a representative sample [16].

We calculated percentages of lymphoblasts, small, medium and large lymphocytes, macrophages, mitotically active cells and damaged cells per 100 cells in the thymic parenchyma, including the subcapsular and inner zones of the cortex and the medulla. Small, medium and large lymphocytes were distinguished based on the morphometric parameters of the nuclear area. According to Kriventsov [17], lymphocytes with the nuclear area of 6 to 14 μm^2 are classified as small, 14 to 22 μm^2 are considered medium and 22 to 30 μm^2 are large.

Data were statistically processed using Student's t-test ($p < 0.05$). Data distribution was normal. Distribution type was identified using Kolmogorov-Smirnov test. Arithmetic mean and standard error ($M \pm m$) were computed.

The experiment was conducted in compliance with the regulations of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Strasbourg, 1986) and approved by the Bioethics Committee of Lugansk State Medical University (Protocol no.1 dated January 19, 2013).

RESULTS

Age-related involution of the thymus was confirmed by the comparative histological analysis of the thymus of the controls and the pubertal rats (those data were obtained prior to this experiment [18]).

Thymic lobules looked smaller than in younger animals (fig. 1). They were separated by thick connective tissue septa. The border between the cortex and the medulla was blurred; medulla size was increased. Lobule parenchyma was partially replaced with white adipose tissue. Similar age-related changes in the thymus of 12-month old rats were described by Moroz [19]; similar changes in 6–10-month old rats were described by Moskvichev et al. [6].

Morphologically, the thymus of the experimental animals did not differ from that of the controls throughout the experiment. We observed capsule- and septa-forming connective tissue overgrowth and partial replacement of the parenchyma with adipose tissue. But at a higher magnification, microscopic images revealed changes in the parenchyma cell profile.

The stroma of the subcapsular zone of the cortex parenchyma is formed by a network of epithelial reticular cells and macrophages. In the stromal area, several layers of round lymphoid cells were observed. The majority of those cells were small and medium lymphocytes, but large lymphocytes and lymphoblasts were also present. Mitoses were rare. Epithelial reticular cells were flat and irregularly shaped, larger than lymphocytes and had a paler cytoplasm. Macrophages were large, irregularly shaped with branching projections and a typical foamy cytoplasm. Damaged lymphoid cells were also observed (those contained hypercondensed chromatin in the shrunk nucleus). We observed an increased number of lymphoid cells and macrophages, compared to the controls, and a reduced number of damaged cells (fig. 2). However, the figures were statistically significant only for the animals sacrificed on days 7, 15 and 30 after the final injection of Imunofan. The number of epithelial reticular cells did not differ significantly from that of the controls in any of the experimental subgroups.

The inner zone of the cortex demonstrated a maximum cell density with several layers of medium and small lymphocytes, some of them mitotically active, integrated into the network

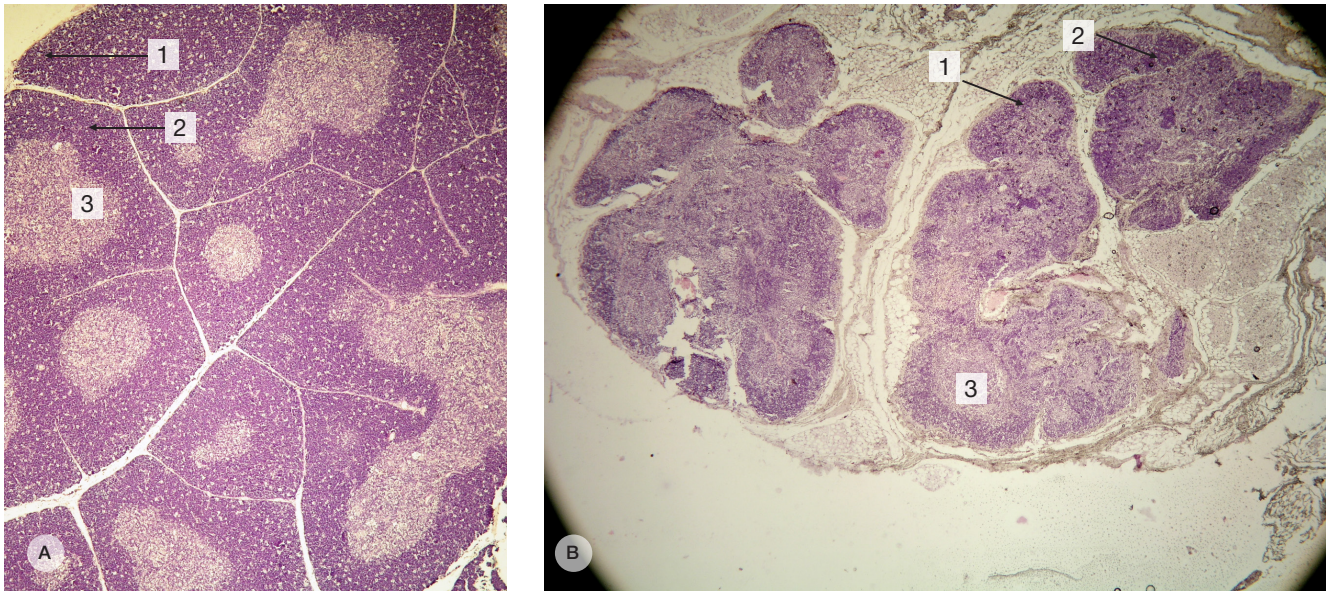

Fig. 1. Age-related involution of the thymus in random-bred albino male rats

Microphotographs of thymic sections obtained from pubertal male rats **(A)** and 20-month-old rats **(B)** one day after the administration of sodium chloride solution. 1 — the subcapsular zone of the cortex; 2 — the inner zone of the cortex; 3 — the medulla. Staining: hematoxylin-eosin. Objective lens: PlanC N x10/0,25 ∞/–/FN22. Zoom 132.

formed by epithelial reticular cells and macrophages. Lymphoid cells with degrading nuclei and cytoplasm were also observed. Changes in the cell profile here were similar to those in the subcapsular zone. One day after the administration of Imunofan, the number of medium lymphocytes increased by 8.5 % and the number of damaged cells dropped by 48.0 % ($p < 0.05$) (fig.3). After decapitation performed on day 7, an increased number of medium and, small lymphocytes, mitotically active cells and macrophages was observed, while the number of epithelial reticular stromal cells and damaged cells dropped by 15.8 and 62.2 %, respectively. On day 15, the same trend was observed as during the previous week, but the increase in the number of medium lymphocytes was no longer statistically significant. On day 30, the number of small lymphocytes and macrophages was higher, compared to the controls (by 11.3 and 51.3 %, respectively). There were 14.9 % less epithelial reticular cells and 23.1 % less damaged cells, compared to the controls. We found no statistical difference in cell profiles on day 60 after the final injection.

The density of lymphoid cells (mainly small and medium lymphocytes) in the medulla was reduced, compared to the cortex. We observed a thicker network of epithelial reticular cells grouped as strands and clusters. We found no lymphoblasts and large lymphocytes in the medulla; the number of medium lymphocytes was significantly higher on days 7, 15 and 30 of the observation (by 6.1, 9.3 and 7.5 %, respectively). The number of small lymphocytes remained increased on days 7 (8.6 %), 15 (10.0 %) , 30 (11.0 %) and 60 (10.4 %) of the observation (fig. 4). The number of mitotically active cells was insignificantly higher only on day 7 of the observation, compared to the controls. The number of damaged cells was significantly lower on days 15 and 30. The difference between the number of epithelial reticular cells and macrophages was insignificant.

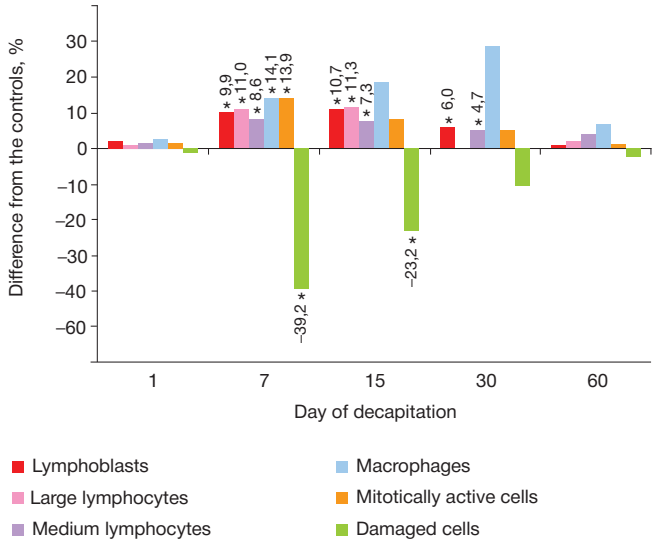

Fig. 2. Cell profile of the subcapsular zone of the thymic cortex of aging rats at various time points after injections of the immunomodulatory agent Imunofan

* — $p < 0.05$ when comparing the experimental and the control groups

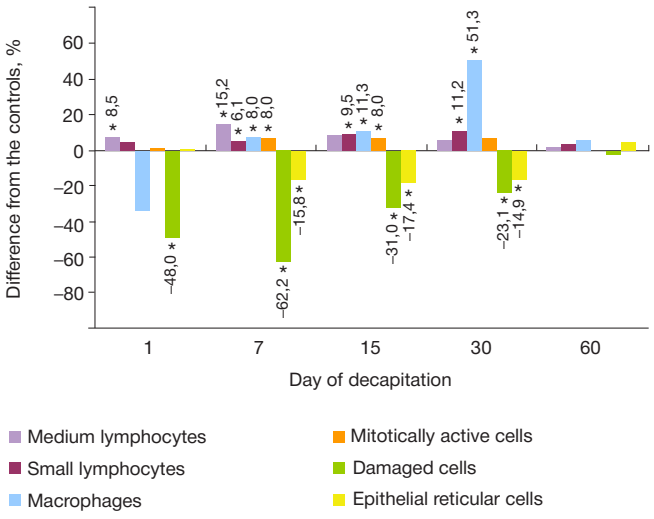

Fig. 3. Cell profile of the inner zone of the thymic cortex in aging rats at various time points after injections of the immunomodulatory agent Imunofan

* — $p < 0.05$ when comparing the experimental and the control groups

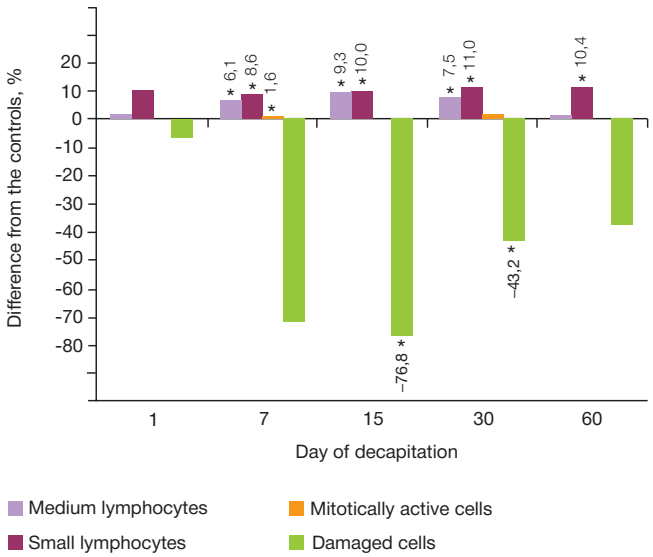

Fig. 4. Cell profile of the thymic medulla in aging rats at various time points after injections of the immunomodulatory agent Imunofan

* — p <0.05 when comparing the experimental and the control groups

By the end of the experiment (day 60), the number of small and medium lymphocytes, young cells, mitotically active cells and macrophages decreased and was about the same as in the controls.

RESULTS

The obtained results indicate a conspicuous thymic response to the administration of Imunofan in aging rats. Changes in cell profile of the subcapsular and inner zones of the thymic cortex were similar, which indicates that cytoarchitecture of the whole cortex was affected. The medulla is the most areactogenic zone of the thymic parenchyma [17], but its cell profile also changed in the experimental group. The possible mechanism of immunostimulating action of Imunofan (including temporary partial delay of age-related involution) can be associated with maintaining and/or restoring lymphoid cell population and its microenvironment, including macrophages. The number of epithelial reticular cells in the subcapsular cortex and the medulla did not differ significantly, which leads us to conclude that Imunofan does not suppress thymic reserve capacities.

Changes to the cytoarchitecture of the thymic parenchyma, namely, a higher cell population density in all thymic zones studied during the experiment and an increased number of lymphoid cells, including young cells, can indicate a more active inflow of precursors from the red bone marrow into the thymus and increased proliferation of lymphocytes. Such stimulating effect on the thymus was observed by some authors when using another immunomodulator, Polyoxidonium [4, 6]. Zakharov [3] describes slower age-related involution in the thymus of adult laboratory rats administered to Imunofan.

CONCLUSIONS

The thymus of aging rats was highly responsive to the administration of Imunofan. Injections of therapeutic doses of the drug contributed to the restoration of the thymic structure and delayed age-related involution of the thymus. A close interaction of various cellular components of the thymus indicates the importance of studying the balance between thymic lymphocytes and their microenvironment treated with immunomodulatory agents.

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