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ONTOGENETIC PATTERNS OF MICROARCHITECTURAL MODULATIONS IN THE SPLEEN OF THE GROWING AND AGING RATS

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Abstract:

Spleen is the largest lymphoid organ involved in cellular and humoral immune response. It becomes structurally and functionally mature in early life, undertakes dynamic age-dependent modulations and undergoes involution during aging of the body. In many current immunological research works developmental modifications in the spleen at different ages remain underestimated, while they may overlap with the changes caused by modelled immunopathology which will result in misinterpretation of data on the effectiveness of immune response, sensitivity to immunomodulatory treatments, etc. While age-related thymic involution is described in details, patterns of ontogenetic immunomodulation in spleen at different ages were hardly updated since last century. The objective of this research is to provide a comprehensive picture of splenic development at different stages of postnatal development and to assess possible mechanisms of the age-related splenic involution. Total of 80 Sprague Dawley rats were involved in the study; out of them 54 were used for digital morphometry being divided into 9 subgroups with 6 rats each: 0, 10, 20, 30, 60, 90, 180, 270 and 360 days of age. Another 26 animals were used for qualitative assessment of the splenic microstructure with 2 animals per group of every day of early life, starting from the 2nd day until the 29th day. Each animal and its spleen were weighed, relative weight of the spleen was estimated. Histological slides of the paraffin-embedded tissue were stained with haematoxylin-eosin and immunohistochemically for markers of the lymphoid and stromal cells (CD3, CD4, CD8, CD20, CD90, S100 protein, OX-62), PCNA and caspase-3. Image analysis was used to assess volume density of the immunopositive structures. Our results demonstrated that developmental changes in the spleen of the growing rats occur faster than it was described in classical papers on postnatal spleen development over fifty years ago: primitive PALS appear on the 2nd day of life, marginal zone – on the 8th day, primary lymphoid nodules – on the 18th day, secondary lymphoid nodules – on the 24th day. Lymphoid cells depletion becomes significant in aging rats, while stromal cells significantly reduce their volume density starting from the middle age. Among mechanisms of age-related lymphoid and stromal cell depletion are increased apoptotic rate, decreased proliferative rate and reduced traffic of T-cells in the T-zones of spleen. These data may be used for discrimination of the developmental and pathological changes in the compartments and zones of the spleen in experiments with modeling of the lymphoid tumors, evaluation of the efficacy of the immunomodulatory drugs, transplantation and stress-related experiments.

Key words: *spleen, age, senescence, involution, immunohistochemistry*

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INTRODUCTION:

Immune system is known to undergo fast developmental changes during prenatal and early postnatal ontogenesis; it reaches maturity faster than other body systems and starts aging earlier than them [1,2]. Age-related thymic involution, which is studied in details [3,4,5**],** changes conditions of functioning of the peripheral immune organs which need to adjust to the declining thymic output and develop adaptive histophysiological changes to provide an effective immune response [6]. Understanding of age-related modulational changes in the peripheral lymphoid organs in general and in the spleen as the largest immune organ in particular is required to promote development of methods of control of the efficiency of the immune response during infections, neoplasms, stress, transplantation of the organs and tissues in patients of certain age [7,6**].**

Spleen contributes to both cellular and humoral immunity, it possesses a unique highly compartmentalized microarchitecture with a specific environment for the lymphoid cells in every compartment, sub-compartment and zone, and unique mode of stromal-parenchymal crosstalk, providing an effective immune response. It is well-established that almost all cellular components of innate and adaptive immunity undergo age-related remodelling [8**].** Deep knowledge of the age-related changes in the spleen may help to better understand the patterns of ontogenetic modulations of the immune response in every age group [9**].**

Though spleen is a highly compartmentalized organ, most of research on spleen were performed using immunological and biochemical methods, and much less – histological ones, which can provide information on modulational changes in the compartments and zones of the organ [8,10**].**

This problem is gaining importance in view of rapid development of the tissue and organ transplantation technologies, which require to conduct immunosuppressive therapy, including splenectomy, taking into consideration age-related condition of the immune organs [11,12**]**. So far to our best knowledge comprehensive description of age-related changes in the microstructure of spleen, covering all age periods and using quantitative immunohistochemical methods is missing.

The objective of this research was to provide a comprehensive picture of splenic development at different stages of postnatal life and to assess the mechanisms of the age-related splenic involution.

MATERIAL AND METHODS:

Sprague Dawley rats aged from age 0 to 360 days were enrolled in this study. This research was approved by the ethical committee of the Faculty of Medicine, UiTM, Selangor, Malaysia, protocol ACUC 4-11, 14.04.11.

The animals were divided into 3 groups: preweaning (PW), juvenile/young (PY), and mature/aging (MA). Each of these groups was further subdivided into 3 subgroups which included the rats aged 0, 10 and 20 days (preweaning group), 30, 60 and 90 days (infant, juvenile and young subgroups) and 180, 270 and 360 days (mature and aging subgroups) with 6 rats per subgroup. Besides, for qualitative splenic studies we used 2 animals of each day of life from the 2nd until the 29th. Upon reaching the corresponding age, the animals were weighed and sacrificed by decapitation under anesthesia. Their spleens were sampled, weighed, processed and embedded in paraffin. Histological sections were stained by H & E and immunohistochemically for CD3, CD4, CD8, CD90, CD20, S100 protein, OX-62, caspase-3 and proliferating cells nuclear antigen (PCNA) using commercial streptavidin-biotin-peroxidase-DAB kits (AbDSerotec, USA) according to recommendations of the manufacturers. Quantitative assessment of the immunohistochemically stained slides was done using an Image Pro+ 8.0 software (Media Cybernetics, US).

All data were presented as the mean ± S.E.M. One-way ANOVA with Student–Newman–Keuls multiple comparison test was applied for statistics; $p<0.05$ was considered statistically significant. Pearson coefficient was used for correlational statistics.

RESULTS:

As shown in the Fig.1, the weight of the animals was increasing very fast in the first two groups and slowed down in the 3rd group. Similarly, the weight of the spleen (Fig.2) was rapidly increasing in all the subgroups of the 1st and 2nd group, but then remained almost at the same level between the 3rd and 6th month of age with subsequent decrease from the 6th month to the end of the 1st year. At the same time relative weight of the spleen started to decrease after the 2 months of life (0.37+/-0.04 g) and continued to decline significantly until the end of the 1st year (0.13+/-0.01 g), indicating at the gross level that splenic involution starts soon after puberty.

Fig.1. Body mass (g) of the growing and aging rats, M+/-m.

Groups/ Subgroups	A	B	C
Preweaning and weaning/ 1st group	4,84 ± 0,51	14,56 ± 1,33***	31,64 ± 3,16***
Prejuvenile and young/ 2nd group	70,83 ± 6,05	228,98 ± 20,74***	320,98 ± 29,95***
Mature/aging 3rd group	380,19 ± 35,74	450,52 ± 39,06	521,06 ± 47,01*

* - p<0.05 compared to the youngest subgroup
*** - p<0.001

Fig 2. Spleen mass (g) of the growing and aging rats, M+/-m.

Groups/Subgroups	A	Б	B
Preweaning and weaning/ 1st group	0,023 ± 0,002	0,068 ± 0,006***	0,104 ± 0,009***
Prejuvenile and young/ 2nd group	0,310 ± 0,052	0,726 ± 0,065***	0,913 ± 0,088***
Mature/aging 3rd group	0,891 ± 0,091	0,754 ± 0,074	0,686 ± 0,070

*** - p<0,001 compared with initial A group

Microscopic evaluation of the histological slides (Fig.3a,b) showed that in the newborn rat pups spleen contains only non-compartmentalized red pulp which appears as an immature haemopoietic tissue with numerous loci of myelopoiesis. On the 2nd day the concentric accumulations of lymphoid tissue appear around the arterioles – the immature periarterial lymphoid sheaths (PALS); by the 8th day marginal zone around PALS becomes visible which looks like a border between the white and red pulp, with subsequent development of the marginal sinus. By the 18th day of life the first lymphoid nodules become identifiable in the white pulp, while the germinal centres appear in them by the 24th day of life. Subsequent modifications in the spleen of the growing rats include relative enlargement of the white pulp and marginal zone, differentiation of the PALS into external and internal zone and intensive dynamics of the lymphoid and stromal cell populations in the splenic compartments, sub-compartments and zones.

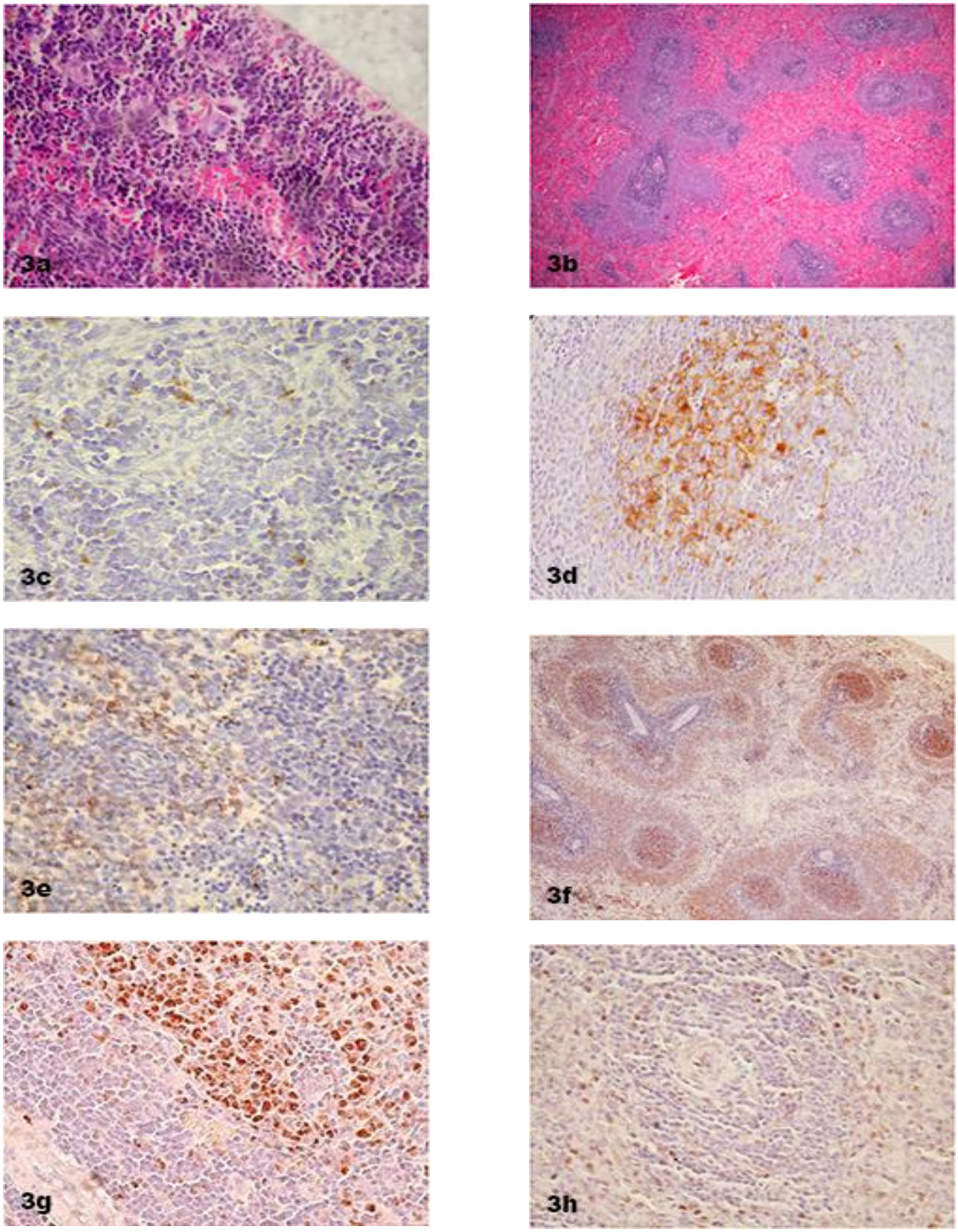


Fig.3. Microphotographs of the spleen of the rats of different age: 0 days (a), 10 days (c,e), 20 days (h), 1 month (b,d), 3 months (f); H & E staining (a,b), protein S100 staining (c,d), CD20 staining (e,f), PCNA staining (g,h). Magnification x400 (c,g); x200 (a,d,e,h) and x40 (b,f).

After birth immunohistochemical staining (Fig.3c-h) showed accumulations of the CD3+, CD8+ and CD4+cells around the arterioles with a diameter 2-3 times wider than the diameter of the corresponding arterioles. Few CD90+ cells were visible in these clusters of lymphoid cells, but their number started to increase from the 2nd day of life. Within these accumulations scarce OX-62 cells were visible. In the meantime, CD20+cells were randomly distributed in the parenchyma of the spleen not forming any clusters. CD20+ cells started collecting in clusters only by the end of the 1st week of life forming irregular aggregates around developing PALS. In the 1st week of life staining for S100-protein was negative, but from the end of the 2nd week (in 12-day old pups) the first accumulations of the follicular dendritic cells became visible, forming the stromal network of the first lymphoid nodules. This network started filling in with the CD20+ cells. During 3rd week (towards the 18th day) this network became denser, while in 19-day old rats it became scant, thus indicating the beginning of development of the germinal centres. Morphometry of the immunohistochemically stained slides confirmed the result of qualitative assessment and provided new details of the age-related changes in the spleen after birth (Fig.4-9).

Fig.4.Volume density (%) of the protein S100+cells in the spleen of the rats of different ages, M±m

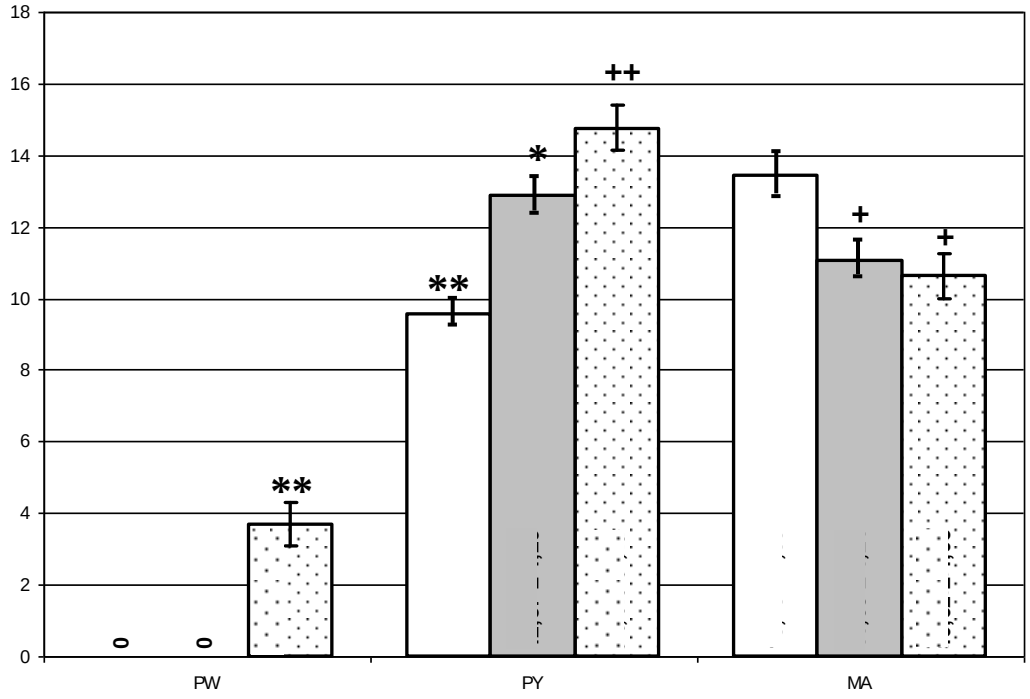


Fig.5. Volume density of the CD20+cells in the spleen of the rats of different age (%), M±m

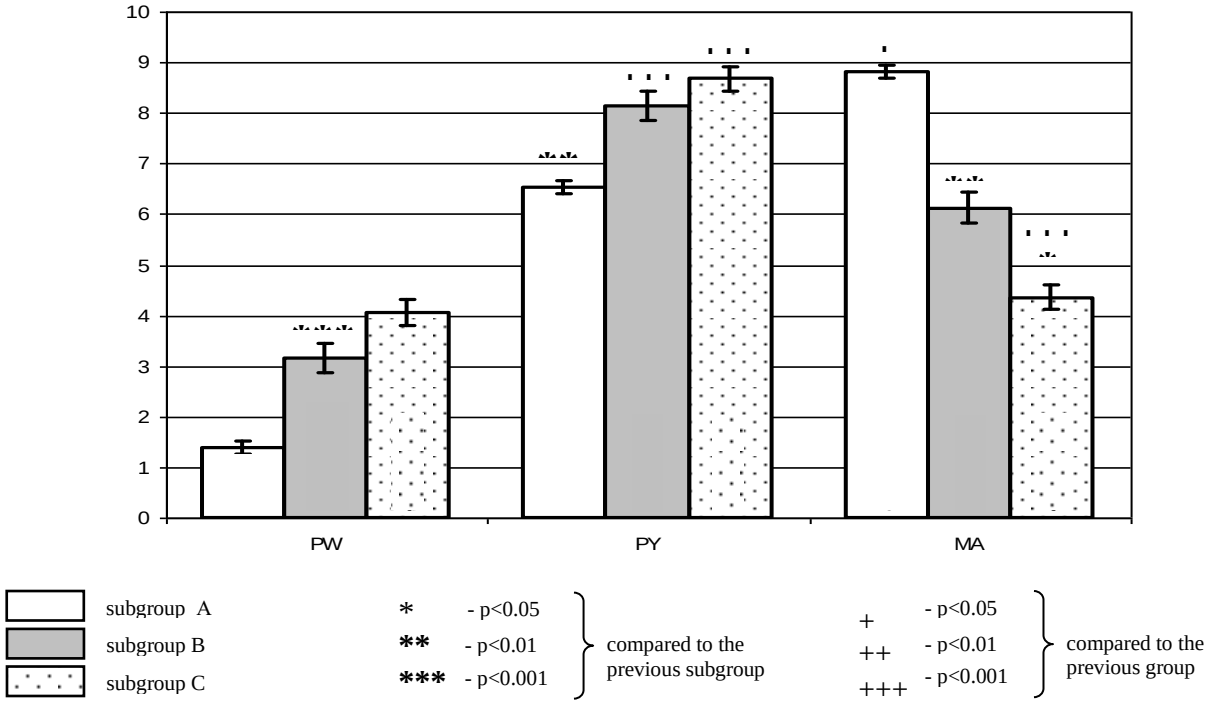


Fig.6. Volume density (%) of the CD8+cells in the spleen of the rats of different age, M±m

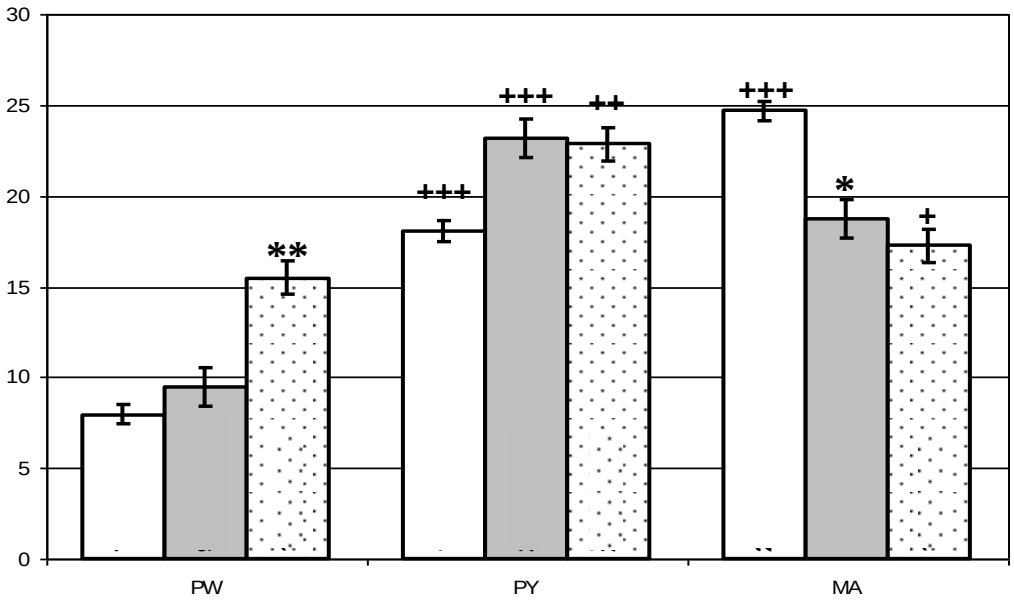


Fig.7. Volume density (%) of the CD90+cells in the spleen of the rats of different age, M±m

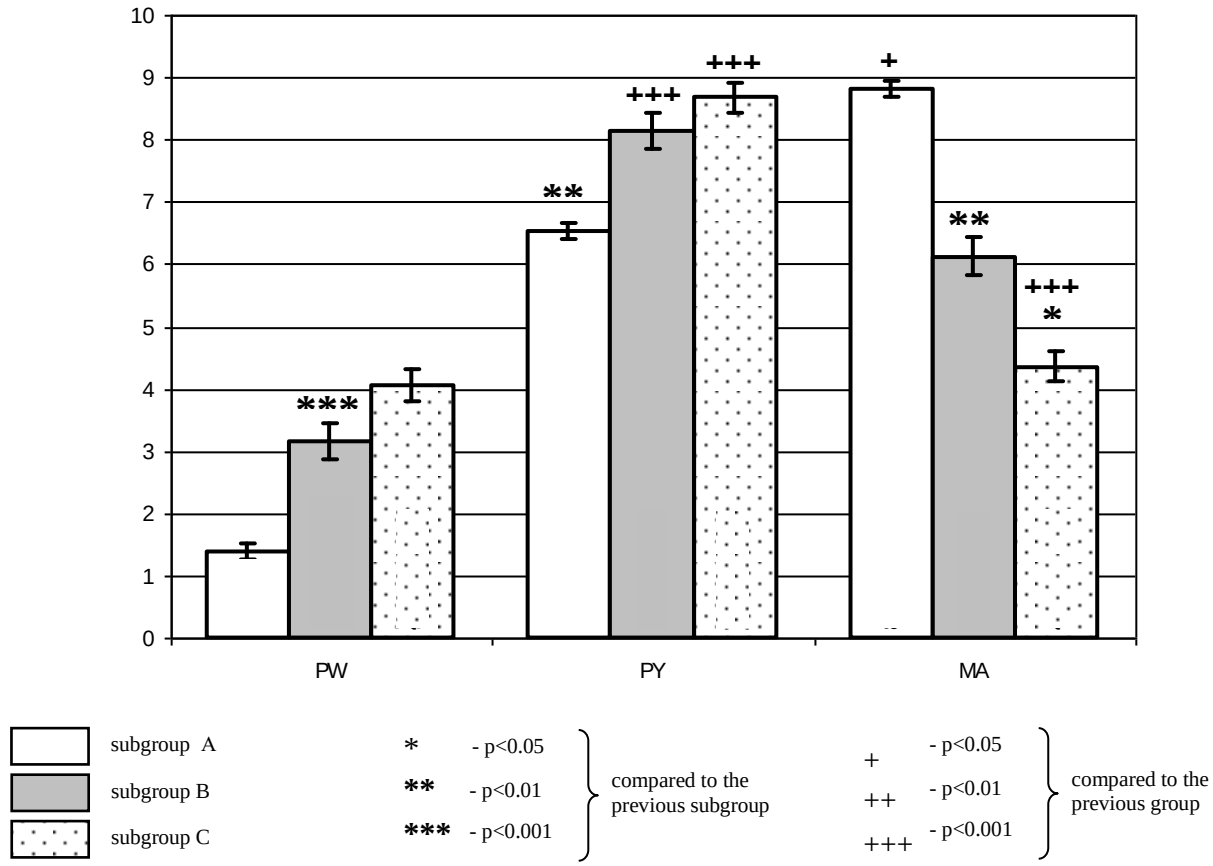


Fig.8. Volume density (%) of the caspase3+cells in the spleen of the rats of different age, M±m

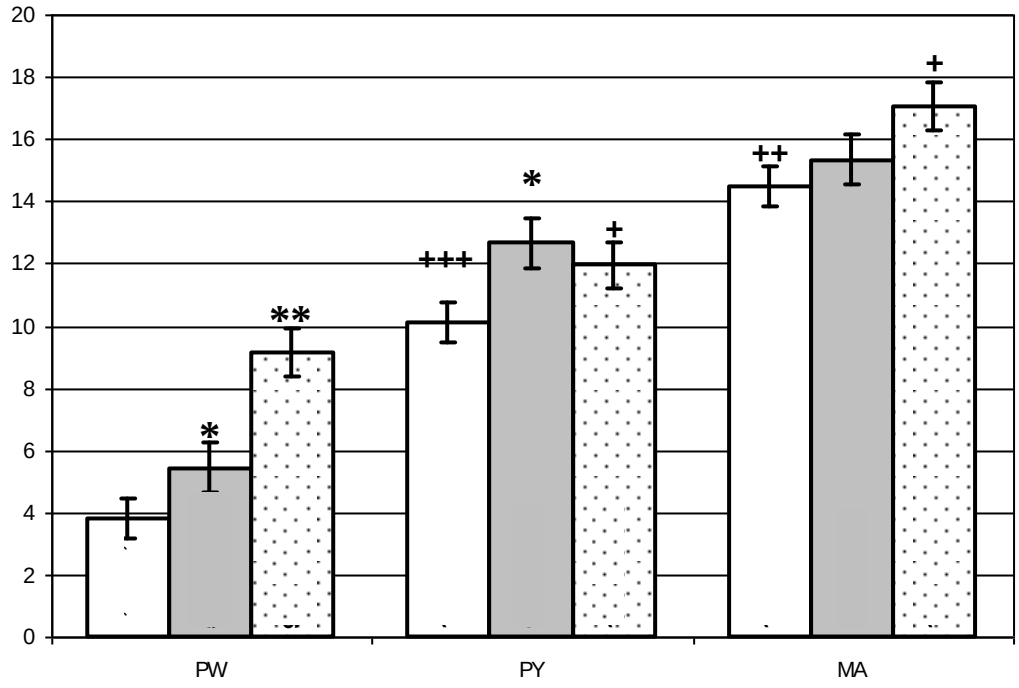
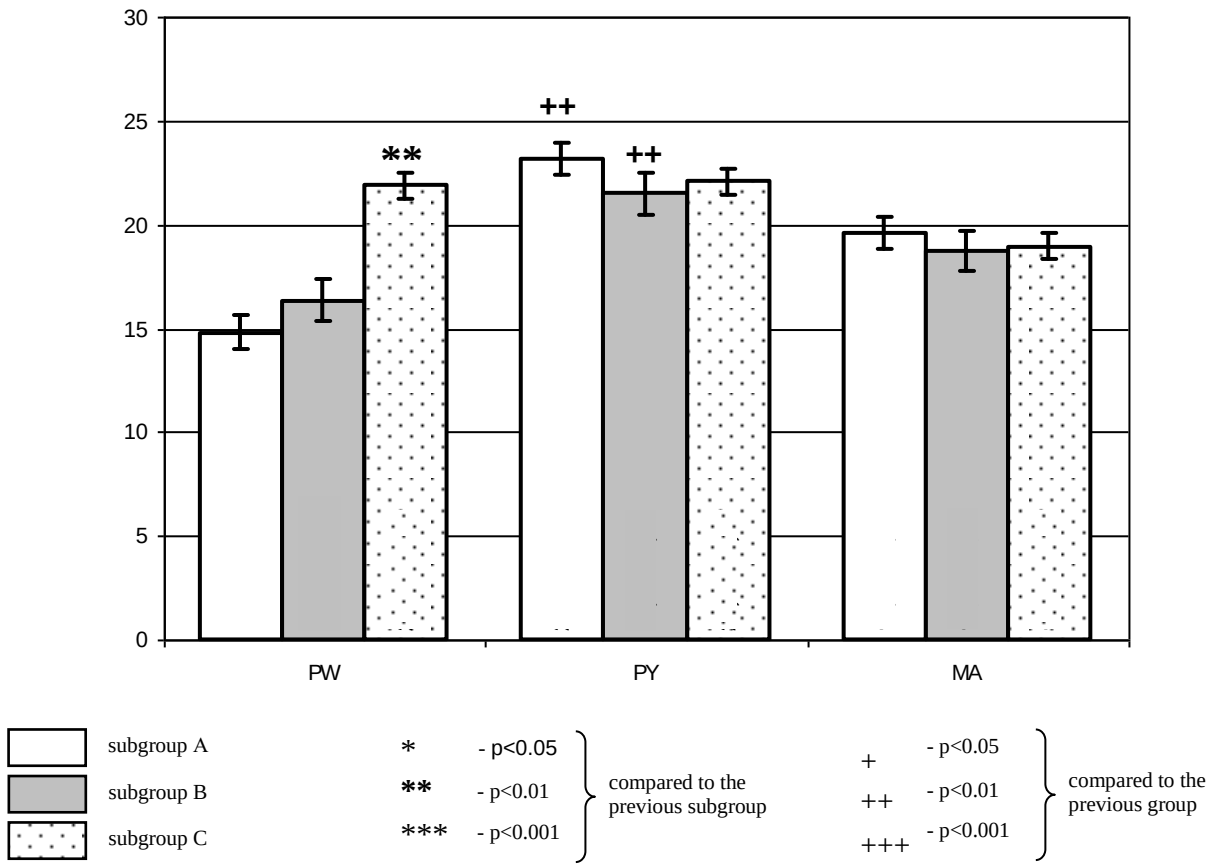


Fig.9. Volume density (%) of the PCNA + cells in the spleen of the rats of different age (%), M±m



S100 protein+ cells were absent in rats of the first two subgroups while they became visible on the 19th day of life and then continued to increase in number until the end of the 3rd month. Then they started to decrease in number, reaching the level of significance in the volume density reduction by the end of the 9th month.

Volume density of the CD20+ cells started to increase rapidly after 10 days of life and continued until the end of the 2nd month. Thereafter this increase slowed down and after 6 months their number started to decrease. This reduction became significant by the end of the 12th month. When we compared age-related dynamics of the S100 protein+ and CD20+ cells, it became obvious that age-related decline of the population of S100 protein+ cells leaves behind a decrease of the volume density of the CD20+cells. Similarly, age-dependent decrease of the OX-62 cells leaves behind the reduction of the volume density of the CD8+ which started after 6 months of life and showed certain parallelism with the age-related dynamics of the CD90+ cells in preweaning and middle-aged rats. Caspase-3+ cells significantly increased their volume density from birth until the end of the 2nd month, thereafter it started fluctuating, reaching its maximum by the end of the 1st year of life. Volume density of the PCNA+ cells reached its highest level by the end of the 1st month, thereafter it started fluctuating showing a trend towards slow decrease. Correlation analysis revealed a strong positive correlation between the volume density of OX-62+ and CD90+cells in the white pulp (r=0,73; p<0,05).

Thus, our results demonstrated certain age-related patterns of the splenic involution and allowed us to discriminate the three periods in the development of the immune architecture of spleen in rats: qualitative evolutional changes in the spleen (preweaning rat pups), the period of quantitative evolutional changes (juvenile/young group) and the period of the quantitative involutional changes in mature/aging group, with overall earlier decline of the stromal cells compared to the parenchymal ones. These results may be used as reference data for the age-related experimental research, such as determination of the target and choice of methods of treatment of immune deficiency or tumors of the lymphoid tissue, which may start at any age and

should get all the age-specific patterns of immunomodulatory changes in the spleen addressed.

DISCUSSION:

Recent research has demonstrated that susceptibility to experimental autoimmune diseases is age-related, and developmental changes in spleen play a big role in the age-dependent level of vulnerability to this pathology. Similar results were shown regarding age-related level of sensitivity to experimental lymphohaematopoietic tumours treatment [10,13]. Therefore, new information regarding ontogenetic changes in the compartment and zones of the spleen of the rats is important for experimental medicine.

First detailed studies of the age-related changes in spleen [14] of the rats aged 1-40 days showed appearance of the lymphoid nodules on the 20th day of life, their germinal centres – on the 25th day, marginal zone – on the 9th day, typical T-zone – on the 14th day; later on the first immunohistochemical investigation of the age-dependent modulations in the spleen were performed [15,16]. For many populations of lymphoid cells there is conflicting information in the literature regarding the effect of aging on them [8,17]. For example, chronological changes in immunohistochemical phenotyping in the spleen in Crl:CD rats up to the age of about one year included the following: white pulp increased until 9 weeks of age and remained fairly stable thereafter; in the periarteriolar lymphoid sheath and marginal zone T cells gradually increased until 9 weeks of age and became almost flat thereafter; in the lymph follicles T cells increased with age; B cells tended towards an increase with age in all areas of the spleen [18**].**

The most significant microstructural manifestations of the age-related modifications in the lymphoid organs are the changes in their compartmentalization, cellularity of their zones which develop due to the alteration of the immunocyte trafficking, decreased level of cell proliferation, increased cell death rate and alteration of the intercellular signalling. At different ages the role of each mechanism may be diverse, which needs to be taken into consideration, when modulation of the immune response is required in patients receiving immunosuppressive therapy or undergoing transplantation of organs and tissues.

Our comprehensive detailed immunohistochemical investigation showed that development of the white pulp in the rat pups continues faster than it was first described in the classical research papers on the micromorphology of the spleen in the growing rats. In our study application of more advanced immunohistochemical methods allowed us to indentify primary lymphoid nodules as early as in 12-day old rat pups, while germinal centres were identifiable in 19-day old rats. Thus, on the contrary to other investigators who believed that compartmentalization of the spleen continues until the end of the 4th week, we demonstrated that qualitatively it is completed by the beginning of the weaning period followed by quantitative modulations indicating its maturation and subsequent involution.

Recently evidence was provided that cooperation of neurological and immune systems is involved in the development of the behavioural disorders in the age-related manner **[19,20**]. Behavioural changes in the neurodevelopmental model of schizophrenia in rats accompanied by alterations in proliferative activity of splenocytes and pro- and anti-inflammatory cytokine levels also revealed age-dependent pattern **[21].** In spite of all these findings, in many experimental research works evaluation of the lymphoid tissue in conducted without looking into the age-related changes of the microstructure of the immune organs which may overlap with the pathological changes and result in wrong conclusions regarding efficacy of the immunomodulating therapies, immunosuppressive effect of stress and other interventions, etc. Therefore, we undertook this study to update the information regarding age-related changes in the largest immune organ – spleen – using immunohistochemistry and image analysis. Our results may be used as reference data in experimental immunomorphological research on age-related issues of application of the new immunomodulators, modelling of immunopathological condition and evaluation of the efficiency of the immune response during infections, malignancies and organ transplantation.

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