UNIVERSITI SAINS MALAYSIA

PPSP, ANATOMY DEPARTMENT

Age-Related Changes in the Immune System of Growing Rats under Acute and Chronic Stress Conditions

USM Short Term Grant Project

(August 1, 2002 – July 31, 2003)

PM Dr *Marina Kapitonova*

PM Dr *Othman Mansor*

PM Dr *Muzammil Ullah*

Kubang Kerian, Kelantan, 2003

BAHAGIAN PENYELIDIKAN & PEMBANGUNAN CANSELORI
UNIVERSITI SAINS MALAYSIA

Laporan Akhir Projek Penyelidikan Jangka Pendek

1) **Nama Penyelidik: PM Dr Marina Kapitonova**

Nama Penyelidik-Penyelidik Lain: PM Dr Othman Mansor,
(*Jika berkaitan*)
PM Dr Muzammill Ullah

2) **Pusat Pengajian/Pusat/Unit: PPSP, Anatomy Department**

3) **Tajuk Projek:**
Age-related changes in the immune system of growing rats under acute and chronic stress conditions.

BAHAGIAN PENYELIDIKAN
PUSAT PENGAJIAN SAINS PERUBATAN

SALINAN :
- [ ] Dhg. Penyelidikan, PPSP
- [x] Perpustakaan Perubatan, USMKK
- [ ] RCMO

T/Tangan : ..... Tarikh : 13.10.03

4. (a) **Penemuan Projek/Abstrak**

(Perlu disediakan makluman diantara 100-200 perkataan di dalam Bahasa Malaysia dan Bahasa Inggeris, ini kemudiannya akan dimuatkan ke dalam Laporan Tahunan Bahagian Penyelidikan & Pembangunan sebagai satu cara untuk menyampaikan dapatan projek tuan/puan kepada pihak Universiti.)

Terdapat banyak kesan stres (tekanan) keatas fisiologi tubuh manusia, salah satunya adalah terhadap sistem imuniti badan. Tidak banyak kajian dilakukan setakat ini terhadap kesan stress keatas sistem imuniti pada peringkat pembesaran. Kajian yang dilakukan setakat ini masih lagi kontroversi.

Kami membuat kajian mengenai kesan stres keatas organ immuniti badan pada peringkat penyusuan (preweaning), selepas penyusuan (post weaning), 'infant' dan 'juvenile'. Dalam kumpulan ekspirimen, tikus Sprague-Dawley dibahagikan kepada 4 kumpulan mengikut usia (14 hari, 21 hari, 30 hari dan 45 hari). Terdapat 8 tikus dalam satu kumpulan. Kumpulan tikus ini dikenakan akut stres (5 jam stres untuk satu hari) ataupun kronik stres (5 jam stres selama 7 hari). Kumpulan ekspirimen ini di bandingkan dengan kumpulan kontrol yang mengandungi 6 ekor tikus dalam satu kumpulan. Organ immuniti timus dan spleen di ambil dan dilabel dengan imunohistokimia spesifik untuk CD3, CD8, CD90,CD45R, ED1 dan capase-3.

Keputusan menunjukkan akut stres dan kronik stres memberikan kesan yang berbeza keatas organ immuniti pada peringkat pembesaran. Akut stres memberikan kesan yang lebih keatas splenik T zone. Manakala dalam kronik stres kedua-dua T zone dan B zone terlibat dalam proses 'immunomodulation', dengan B zone terlibat dalam proses immunosupression dan T zone terlibat dalam proses adaptation. Kesan stres keatas sistem imuniti (imunomodulation) didapati bergantung kepada umur haiwan tersebut. Kesan imunomodulation adalah lebih ketara keatas haiwan pada peringkat penyusuan (weaning). Ini adalah berbeza daripada kesan yang dilaporkan keatas haiwan dewasa, mencadangkan adaptasi stres pada peringkat perkembangan adalah unik dan berbeza daripada peringkat dewasa.

Stress results in a wide range of physiological responses in the body including suppression of immune function. Only few studies evaluate stress-induced changes in the immune system of the growing body, and the results reported for different ontogenetic stages are controversial.

We have evaluated the impact of stress on the immune organs of preweaning, postweaning, infant and juvenile animals. Four experimental groups of prepubertal Sprague-Dawley rats (14, 21, 30 and 45 days old, 8 animals per group) were exposed to either acute (one 5-hour session) or chronic (seven 5-hour daily sessions) restraint stress and assessed against age-matched control groups (6 rats per group). Thymuses and spleens were examined using routine histological and immunohistochemical staining for CD3, CD8, CD90, CD45R, ED1, and caspase-3.

Acute and chronic stress differentially affected the immune organs of the growing body. Splenic T-zones were mainly affected under acute stress conditions, while in chronic stress both T- and B-zones were involved in immunomodulation, with the B-zones subject to immunosuppression, and T-zones revealing certain adaptation processes. Stress-induced immunomodulation depended on the age of the animal. It was most prominent in the animals undergoing stress during weaning period. These alterations differ from those reported in adulthood suggesting distinct adaptation potential in growing body under stressful conditions.

(b) Senaraikan Kata Kunci yang digunakan di dalam abstrak:

Bahasa Malaysia	Bahasa Inggeris
Stress immobilisasi	Restraint stress
Immunosupression	Immunosuppression
Immunohistokimia	Immune organs
Immunohistokimia	Immunohistochemistry
Timus	Thymus
Spleen	Spleen
Tubuh yang membesar	Growing body

5. Output Dan Faedah Projek

(a) Penerbitan (*termasuk laporan/kertas seminar*)
(*Sila nyatakan jenis, tajuk, pengarang, tahun terbitan dan di mana telah diterbitkan/dibentangkan*)

1.Marina Kapitonova, Othman Mansor. Immunohistochemical Characteristics of Accessory Cells of Spleen in the Growing Albino Rats // In: Proceedings of the 12th FAVA Congress 2002, in conjunction with 14th VAM Congress, **Kuala Lumpur**, August 26-28, p.175.

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2. Marina Kapitonova, Othman Mansor, Muzammil Ullah, Asnisam Asari M. Immunohistochemical Evaluation of the Effect of High and Low Emotion Restraint Stress on Immune and Endocrine System of Prepubertal Rats // **Int. J. Immunorehabilitation"**, 2002, Vol. 4, N2, p.333.

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3. Marina Kapitonova, Othman Mansor, Muzammil Ullah, Asnizam Asari M. Immunohistochemical characteristics of thymus of the growing body under stress conditions // Proceedings of the 8th National Conference on Med. Sci "Medicine in the Genomic Era", 8th-9th May 2003, Universiti Sains Malaysia, p.112-113.

(b) Faedah-Faedah Lain Seperti Perkembangan Produk, Prospek Komersialisasi Dan Pendaftaran Paten

(*Jika ada dan jika perlu, sila gunakan kertas berasingan*)

Publications in the journals; histological sections of lymphoid organs (spleen) and endocrine glands (pituitary) stained immunohistochemically for CD3, CD8, CD20, ACTH, PCNA to be used for teaching histology to the MD-students and postgraduates; digital pictures of the sections of the spleen and pituitary stained immunohistochemically to be used for research training of the postgraduate students.

(c) Latihan Gunatenaga Manusia

i) *Pelajar Siswazah:* the results of the research were presented to the MD students at the lectures on "Cell", "Cell Cycle" and "Connective Tissue" to demonstrate identification of prolife-rating cells, subtypes of the lymphoid and macrophageal cells.

ii) *Pelajar Prasiswazah:* the results of the research were presented to the MSci students at the lectures on Cytology and Immune organs to demonstrate antigenic determinants expressed by T- and B-lymphocytes, NK-cells, dendritic cells and adenocorticotrophs. MSci student Dr Asnizam Asari M. was an active participant of the project and co-authored most of the research presentations and publications on the project.

iii) *Lain-lain:*

6. Peralatan Yang Telah Dibeli: none

UNTUK KEGUNAAN JAWATANKUASA PENYELIDIKAN UNIVERSITI

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TANDATANGAN PENGERUSI
JAWATANKUASA PENYELIDIKAN
PUSAT PENGAJIAN
fn:borang/adlinaimc/nak

reduced. In pre-weaning animals the reduction of the CD8a+ cells in the cortex against control was much more prominent with many apoptotic cells being immunoreactive. These results suggest that under stress conditions pre-weaning rats are more vulnerable than early post-weaning animals in terms of immunosuppression and provide a better understanding of stress-induced alteration of the immune response in the growing body.

ABSTRACT CODE (P - 17)

Title : A COMMON METABOLIC PATHWAY MEDIATING THE EMBRYOTOXIC EFFECTS OF ENDOMETRIOSIS AND INTERLEUKIN-8

Authors : Gregory J S Tan, Liza Noordin and Mohd Shukri Othman***

*Institutions : International Medical University, Bukit Jalil, Kuala Lumpur and the Departments of *Physiology and **Obstetrics & Gynaecology, Universiti Sains Malaysia, Kubang Kerian*

Objectives : To examine whether or not excess pyruvate can provide the metabolic substrate to support the growth of early embryos in the presence of peritoneal fluid with endometriosis or interleukin-8.

Introduction : The adverse effect of peritoneal fluid with endometriosis on early embryo growth has previously been described (1). Recent studies suggest that interleukins may be the embryotoxic factor mediating the effect of endometriosis. This hypothesis is supported by the presence of interleukins in the peritoneal fluid obtained from women with endometriosis (unpublished observation). A common metabolic pathway mediating the effects of endometriosis and interleukin could provide further support to the hypothesis.

Methodology : Two-cell mouse embryos were cultured in 1 ml Whitten's medium as previously described (1). The effects of excess pyruvate (1 mmol) on early embryo growth in the presence of peritoneal fluid with endometriosis or interleukin-8 were examined. The embryos were cultured for 3 days.

Results : Both peritoneal fluid with endometriosis and interleukin-8 were found to significantly inhibit the development of early mouse embryos. The in vitro development from 2 cells to blastocysts was suppressed in the presence of peritoneal fluid with mild, moderate or severe endometriosis ($p<0.001$ Fisher's exact test). Interleukin-8 (16 and 1000 pg/ml) however inhibited mainly the development of the later stage embryo, from morulae to blastocysts ($p<0.001$ Fisher's exact test). Interestingly, pyruvate was effective in reversing the embryotoxic effects of both the peritoneal fluid with endometriosis and interleukin-8.

Conclusion : The ability of pyruvate to reverse the embryotoxic effects of both peritoneal fluid with endometriosis and interleukin-8 suggests that a common pathway may be involved in their actions. This provides further support to the hypothesis that interleukins mediate the embryotoxic effect of peritoneal fluid in endometriosis.

Reference :

1. *Gregory J S Tan, Liza Noordin, Wan Hafizah, Mohd Shukri Othman & M H Rajikin (2002) Embryotoxicity of peritoneal fluid with endometriosis:effects of pyruvate. Proc. 5th Scientific Congress of the Federation of Asian & Oceanian Physiological Society, Kuala Lumpur, 23-26 September 2002, p 302.*

BamHI stop codon

The purified RT-PCR product was ligated to pGEM®-T vector system (Promega, Inc.) and transformed into E. coli JM109. The plasmid DNA was subjected to digestion with BamHI and NdeI, PCR, and DNA sequencing using SP6 and T7 primers. The capsid gene was sub-cloned into pET-3C T7 expression plasmid (Novagen, Inc.) and transformed into E. coli BL21(DE3)pLysS (Promega, Inc.). The clone was induced with 0.5mM IPTG, loaded onto SDS-PAGE, and stained with coomassie blue. RESULTS. A band of 396 bp was amplified and successfully ligated into PCR vector. DNA sequencing result confirmed that the insert was capsid gene that encodes 113 amino acids. A band of 12.5 kDa was detected on SDS-PAGE. CONCLUSIONS. The capsid gene of dengue virus type 2 (New Guinea-C Strain) was amplified and the protein was expressed in bacteria. REFERENCES. 1. Gubler, D. J. 1998. Clin. Microbiol. Rev. 11:480-496. 2. Leyssen, P., E. De Clerco, and J. Neyts . 2000. Clin. Microbiol. Rev. 13:67-82. 3. Marks, R. M., H. Lu, R. Sundaresan, T . Toida, A. Suzuki, T. Imanari, M. J. Hernaiz, and R. J. Linhardt . 2001. J. Med. Chem. 44:2178-2187. 4. Monath, T. P. 1994. Proc. Natl. Acad. Sci. USA 91:2395-2400.

ABSTRACT CODE (P - 16)

Title : IMMUNOHISTOCHEMICAL CHARACTERISTICS OF THYMUS OF THE GROWING BODY UNDER STRESS CONDITIONS

Authors : M.Yu. Kapitonova, Othman Mansor, Muzammil Ullah & M. Asnizam Asari.

Institution : Department of Anatomy, School of Medical Sciences, Universiti Sains Malaysia, Kubang Kerian, Kelantan, Malaysia

Introduction : Stress, both physical and psychological, can activate the hypothalamic-pituitary-adrenal axis, leading to a wide range of physiological responses including increased corticosteroid release and suppression of immune function. Very limited research has been performed to elucidate the effect of chronic physical and psychological stress on immature immune system of the growing body (N.Tarcic et.al., 1995; L.Domingues-Gerpe et al., 1997, 1998; A.Wakikawa et al., 1999; H.Takeuchi et al., 2001; J.Lehmann et al., 2002).

Objectives : To compare the degree of immune suppression in the body of pre-weaning and early post-weaning rats exposed to the chronic restraint stress by immunohistochemical evaluation thymus.

Methodology : Two and three week old Sprague Dawley rats (6 animals in each group) were exposed to the daily 5-hours sessions of restraint stress for seven days continuously with another two groups of intact animals (6 in each group) serving as an age-matched control. The animals were euthanized immediately after the last stress exposure. Thymus, spleen and pituitary gland were removed from each animal, fixed in formalin, embedded in paraffin and processed for routine histology and immunohistochemistry using antibodies against CD3 and CD8a T-cell markers.

Results and Conclusion : Thymic atrophy was shown by reduction of thymic weight and cortex/ medulla ratio in pre- and post-weaning experimental groups with both the lymphoid cells and epithelial stroma affected. In the pre-weaning and post-weaning control groups apoptosis of the cortical thymocytes and tingible body macrophages were rare while in both experimental groups they were common. The proportion of the tingible body macrophages was much higher in the pre-weaning animals than in the post-weaning ones. CD3-immunoreactive cells in both control groups were more frequent in the medulla compared to the cortex. In the post-weaning experimental group animals the amount of CD3+ thymocytes in the cortex was reduced and mainly confined to the subcapsular regions while in pre-weaning experimental group only accidental CD3+ cells could be discovered in the cortex. CD8a+ thymocytes were widely spread in the cortex of the control animals, especially in the subcapsular region and in the inner cortex. In post-weaning experimental animals the number of immunoreactive cells was considerably

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IMMUNOHISTOCHEMICAL CHARACTERISTICS OF ACCESSORY CELLS OF SPLEEN IN THE GROWING ALBINO RATS

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Introduction. The distribution of S-100 protein in normal or reactive lymphoid organs has been described in some of the recent investigations with amazingly controversial results (Y.Atoji e.a., 1991;F.E.Gibbs e.a., 1995; W.Schwaeble e.a., 1995). Most investigators support hypothesis that there are two histiocytic cell lines in the white pulp of spleen: one is the monocyte-macrophage system represented by S-100 negative follicular dendritic cells in the lymphatic follicles creating suitable environment for B-lymphocyes, another is the S100+ T-zone histiocyte system constituting stroma of periarterial lymphoid sheaths (PALS) (G.S. Wood e.a., 1985; T.Satoh e.a., 1997; P.Muretto, 1998). By contrast the other investigators (T.Iwanaga e.a., 1982; D.Coccia e.a., 1983; G.Rowden e.a., 1985 H.Haimoto e.a.,1987) observed S-100 protein immunoreactivity in most of the spleen follicular germinal centers. At the same time S-100 protein was detected in both interdigitating reticulum cells and dendritic reticulum cells of rat and human lymphoid organs (M.Sugimura e.a., 1987; A.Carbone e.a., 1988; W.Schwaeble e.a., 1995).

Objective. The objective of the present research is to investigate the distribution of S-100 protein-positive cells in the spleen of growing albino rats.

Methodology. 15 Sprague Dawley albino rats of prepubertal age were used for the investigation, 5 of them were of preweaning age (14 days old) – 5 animals of weaning age (21 days old) and 5 animals of postweaning age (30 days). The animals were euthanised under chloroform anesthesia, the spleens were removed, fixed in 10% buffered formalin, and embedded in paraffin. Serial section were prepared for hematoxylin and eosin and immunohistochemical examination for S-100 protein. Briefly, after deparaffinization the sections were immersed in methanol-H_2O_2 solution for 30 min to block endogenous peroxidase activity, incubated with primary anti-human antibody for S-100 protein (DAKO, Denmark) for two hours at room temperature in the humid chamber and then were treated using the streptavidin biotin complex method. Coloration was developed in 0.06%DAB solution. Sections were counterstained with hematoxylin-eosin.

Results. In 14-day old rats only PALS may be identified in the white pulp of spleen and they are S-100 protein-negative. By weaning period the first lymphatic follicles appear in the white pulp. In 21-day old animals a fine network composed of dendritic processes with S-100 protein immunoreactivity is discovered in the rare lymphatic follicles of the spleen distributed between non-stained PALS. In 30-day old animals S-100 protein is determined on the surface of cells with dendritic appearance within mantle zone of primary lymphatic follicles whose amount is markedly increased by this age. Very rare secondary lymphatic follicles reveal staining in both mantle zone and germinal center, the latter being more intensively stained. Neither periarterial zone of lymphatic nodules nor PALS displayed staining for S-100 protein. It was also absent at the border between the PALS and the marginal zone. No extrafollicular staining was revealed in the white pulp of rats within all the prepubertal age groups.

Conclusion. These data indicate that in prepubertal rats S-100 protein is confined to the spleen follicular dendritic cells which are exclusively associated with B lymphocytes of the lymphatic follicles and maybe identified immunohistochemically since weaning.

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IMMUNOHISTOCHEMICAL EVALUATION OF THE EFFECT OF HIGH AND LOW-EMOTION RESTRAINT STRESS ON IMMUNE AND ENDOCRINE SYSTEM OF PREPUBERTAL RATS

M.Yu.Kapitonova, M.Othman, Muzammil Ullah, M. Asnizam Asari
Department of Anatomy, School of Medical Sciences, USM, Malaysia;

Stress, both physical and psychological, can activate the hypothalamic-pituitary-adrenal axis, leading to a wide range of physiological responses including increased glucocorticoid release and suppression of immune function. Research examining the effect emotionally-laden stressors may have on corticosteroid release is scarce. Very limited research has been performed to elucidate the effect of physical and emotional stressors on immature immune and endocrine system of the growing body (A.M.Jasnow e.a., 2001; D.Consten e.a., 2002; H.F.Figueiredo e.a., 2001, F.de Groof e.a., 2002; J.Lehmann e.a., 2002; C.Kaur e.a., 2002; V.A. Nejtek e.a., 2002).

In present investigation prepubertal 1-month old Sprague-Dawley rats were exposed either to high or to low emotion acute immobilization stress (stretched on the back or squashed in the tight perforated plastic box respectively). Each group was comprised of 8 animals, the other 6 rats not exposed to stress serving as the age-matched control. Experimental animals received a single 5-hour restraint episode and were sacrificed immediately after stress exposure. Thymuses, spleens, pituitary and adrenal glands were examined using routine histological and immunohistochemical staining.

In the low emotion physical stress-exposed group the ACTH staining of adenohypophysis was less intensive than in the high emotion restraint stress-induced animals, whereas spleen lymphocyte apoptosis was much more frequent in the high emotion stress-administered rats. Increase of lymphocyte cell death rate in spleen was mainly confined to the lymphatic follicles. In both groups both ACTH-staining and spleen lymphocyte apoptosis incidence were significantly higher than in control animals. As pituitary ACTH-staining patterns and incidence of lymphocytes apoptosis in the B-zones of the spleen white pulp differed in low and high emotion restraint stress-exposed animals; it has been concluded that immobilization itself is not the only significant factor in the hormonal and immune response of the growing body to restraint stress.

Age-Related Changes in the Immune System of Growing Rats under Acute and Chronic Stress Conditions.

Short Term Grant Project (August 1, 2002 – July 31, 2003).

Principal investigator: PM Dr Marina Kapitonova
Co-investigators: PM Dr Othman Mansor, PM Dr Muzammil Ullah

Introduction. Considerable experimental evidence has been reported which indicates that stress exerts a negative influence upon a variety of in vivo and vitro immune responses (S.Zalcman et al., 1993; R.L.Spencer et al., 1996; R.Archana et al., 2000; A.Bartolomucci et al., 2001; M.E.Bauer et al., 2001). As it has been shown in some of investigations, organism's ability to respond to stress by modulation of its immune functions depends on different factors, namely age, gender, previous life history, type of stress, duration of exposure etc (L.Dominguez-Gerpe et al., 1998; L H.Takeuchi et al., 2001). By contrast the scant information available regarding the immunological effect of stress in a developing body.

Only few papers deal with age-related changes in the immune system under experimental stress conditions, most of which are concentrated on the comparison of immunomodulation in young and old animals (M.Odio et al., 1987; E.Ortega et al., 1994; K.Takahashi et al., 1997; Dominguez-Gerpe L., 2001). As for post-weaning, infant and juvenile periods of life they have never been investigated in terms of vulnerability of secondary lymphoid organs under stress conditions. At the same time it is well known that during infancy immune system is most vulnerable to many infections especially those with encapsulated bacteria such as Haemophilus influenzae B,

Streptococcus pneumoniae and Neisseria meningitides as antibody responses to bacterial capsular polysaccharides (type II thymus-independent responses) develop only after infancy and that worldwide some five million children aged less than 5 years die annually from infections with encapsulated bacteria (C.G.de Vinuesa et al., 1999).

Some studies indicate that stresses result eventually in either suppression or enhancement of the immune system in animals and men (J.F.Sheridan et al., 1991; T. Kizaki e.a., 1995 N.Shanks et al., 1998; Oya et al., 2000). While one group of investigators describes less severely suppressed immune function in acute than in chronic stress (R.L.Spencer et al., 1996), others believe that repeated stress potentiates immune response (S.Zalcman et al., 1993). The elucidation of the range of the immunomodulating changes and mechanisms involved in it is of interest for the design of therapeutic approaches to avoid appearance of stress disorders.

A study of structural changes in primary and secondary lymphatic organs provides an adequate assessment of immune status (C.Gopinath, 1996). Spleen proved to be the most highly compartmentalized immune organ with tight distribution of the immune response constituents between its subcompartments: white pulp, red pulp and marginal zone. In each of the subcompartments certain domains are distinguished (lymphatic nodules and periarterial sheathes which are in turn subdivided into distinct zones). Assessment of changes within different compartments and subcompartments of the spleen will provide valuable information on deviations of immune response under acute and chronic stress conditions in the developing organism. Many authors state usefulness of immunohistochemistry as an aid in estimation of immunosuppressive response but differential effects of

stress across spleen subcompartments, domains and zones using image analysis and immunohistochemistry have never been assessed before.

The objectives of the present investigation therefore were to obtain qualitative and quantitative characteristics of spleen compartments in growing rats using modern methods chemistry and image analysis, to evaluate the impact of acute and chronic immobilization stress on immunomorphology of the secondary immune organs (spleen) of rats in different age periods (pre-weaning, weaning, infant, pre-juvenile).

Material and Methods. One hundred four prepubertal albino rats of 4 age groups (preweaning – 2 weeks old, weaning – 3 weeks, infant – 4 weeks old, and prejuvenile – 6 weeks old) were either subject to acute or chronic stress (immobilization for 5 hours on the back for 1 or 7 days respectively) or used as an age-matched control. Within each age group of the animals three experimental sub-groups were discriminated with 6 animals per group and two control groups (to acute and chronic stress experimental group accordingly) with 4 animals per group. The rats of the 1st experimental sub-group were exposed to acute restraint stress, the animals of the 2nd group were exposed to acute stress with preliminary 1 intraperitoneal injection of metapyrone (100 mg per kg of weight), the rat pups of the 3rd group were exposed to chronic stress. The 1st control group served as an age-matched control to the rats of the 1st and 2nd experimental groups (subject to acute stress), and the 2nd control group was assessed against 3rd experimental group (exposed to chronic stress). After the experiment was over, the animals were weighed, anesthetized with chloroform and sacrificed by decapitation; spleen, thymus and pituitary were removed, weighed and fixed in formalin.

Routine histological procedures. Fixed tissues were dehydrated and imbedded in paraffin using Tissue processing center – Tissue-Tek (Sakura) and Tissue Embedding Center "Thermolyne". Sectioning was carried out on the Thermo Shandon (Finesse) microtome with disposable blades; 3-4 micron thick sections were stained with haematoxilin and eosin using automatic staining center "Sakura Tissue Tek DRS". For immunocytochemical procedures sections will be mounted on the lysine-covered glasses.

Immunocytochemistry. Sections of the spleen and thymus were stained for fractions of mononuclear cells in the white and red pulp of spleen, its marginal zone, the cortex and medulla of thymus with the following antibodies: mouse anti-rat CD8a (Pharmingen, clone OX-8), mouse anti-rat CD90 (Pharmingen, clone HIS51), mouse anti-rat CD45RC (Serotec, clone OX22), CD45R (Pharmingen, clone HIS24), mouse anti-rat CD3 (Serotec, clone 1F4), mouse anti-rat B cell (Serotec, clone RLN-9D3) – for lymphoid cells, mouse anti-rat ED1 (Serotec), rabbit anti-cow S100 (DAKO, with cross-reactivity to rat tissues) – for macrophageal cells. Staining was performed using ABC-peroxidase method. Briefly, test slides and appropriate controls were dewaxed, dehydrated and immersed in a fresh 3% solution of hydrogen peroxide in methanol for 15 minutes in order to block endogenous peroxidase activity and rinsed in water and placed in TBS. For most immunohistochemical reactions (except S100) a microwave treatment in the citric buffer pH6.0 for 4 minutes for the retrieval of the antigen was required with further placement in TBS. Sections were incubated with the primary antibodies diluted with Tris-buffered saline (TBS) for 60 min in the immunostainer. An ABC streptavidin-peroxidase method was used to detect immunoreactivity of the tissues. Incubation with primary antibody was

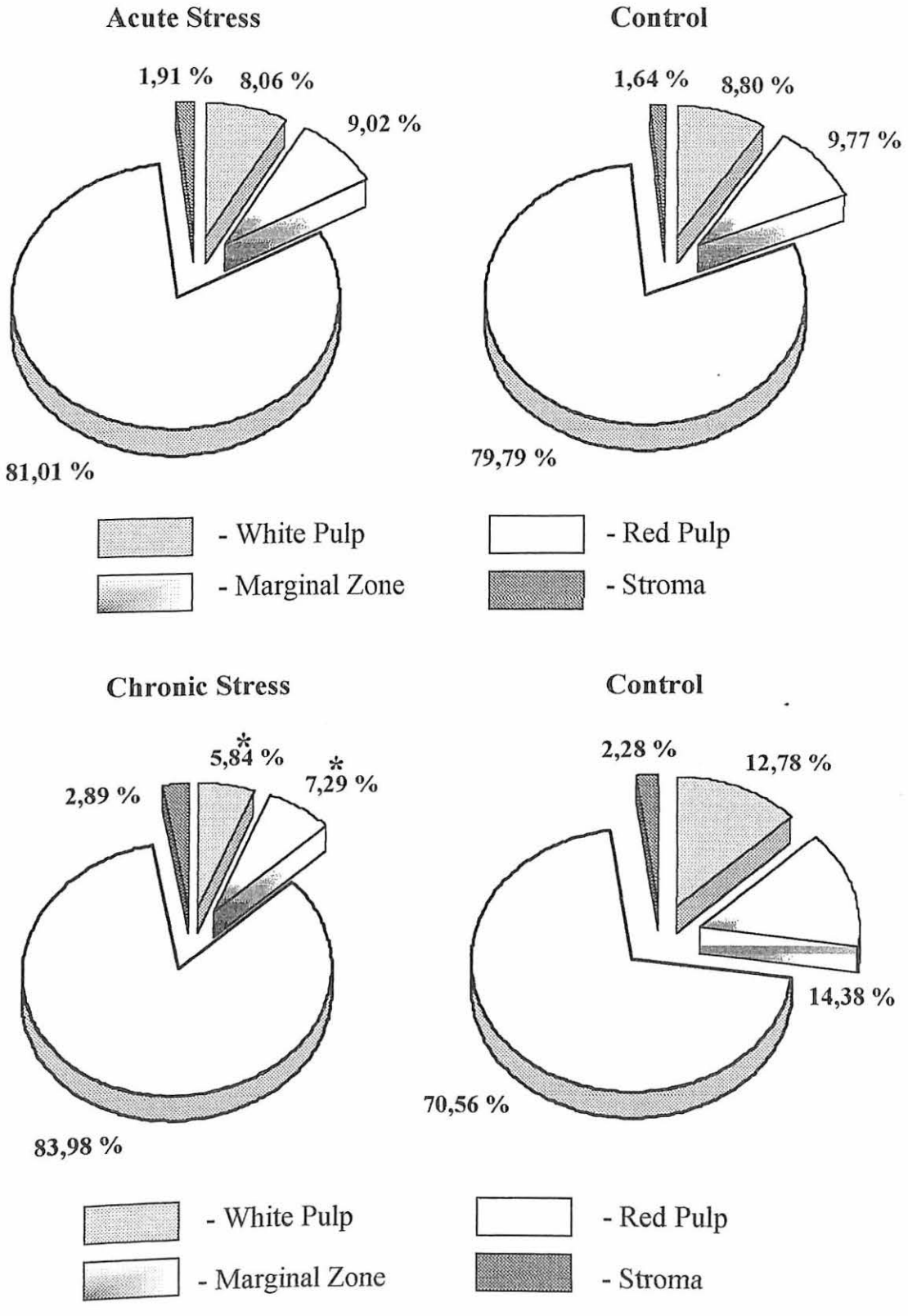
followed by incubation with biotinylated gout anti-rabbit secondary antibody diluted 1/300 with TBS and streptavidin – peroxidase complex for 30 min each. Between each of the steps described above, slides were rinsed in TBS for 30 min. Following a final 30-min rinse, they were immersed in 1M Tris pH 7.4 for 2 min, then developed in 0.05% diaminobenzidine with 0.03% hydrogen peroxide.

Image analysis. Digital pictures of sections obtained with digital camera Nikon COOLPIX 995 were subject to computer-aided quantitative assessment of the changes in the spleen subcompartments, domains and zones (marginal zone, lymphatic nodules and their germinal centers, central arteries, periarterial zones, mantle zones; pulpar arteries, periartherial lymphoid sheathes (internal and external domain), capsule and trabecules, red pulp. In thymus cortex and medulla of the lobules will be assessed. Mean area, perimeter, max and min diameter and their ratios, form-factor were be evaluated for each zone and domain. Original software TRIM was applied for computer-aided analysis.

Statistical analysis included estimation of the mean values, standard deviation, mean error, t- Student's coefficient, Smirnov-Kholmogorov test (for non-parametric distribution where necessary) for comparative statystics.

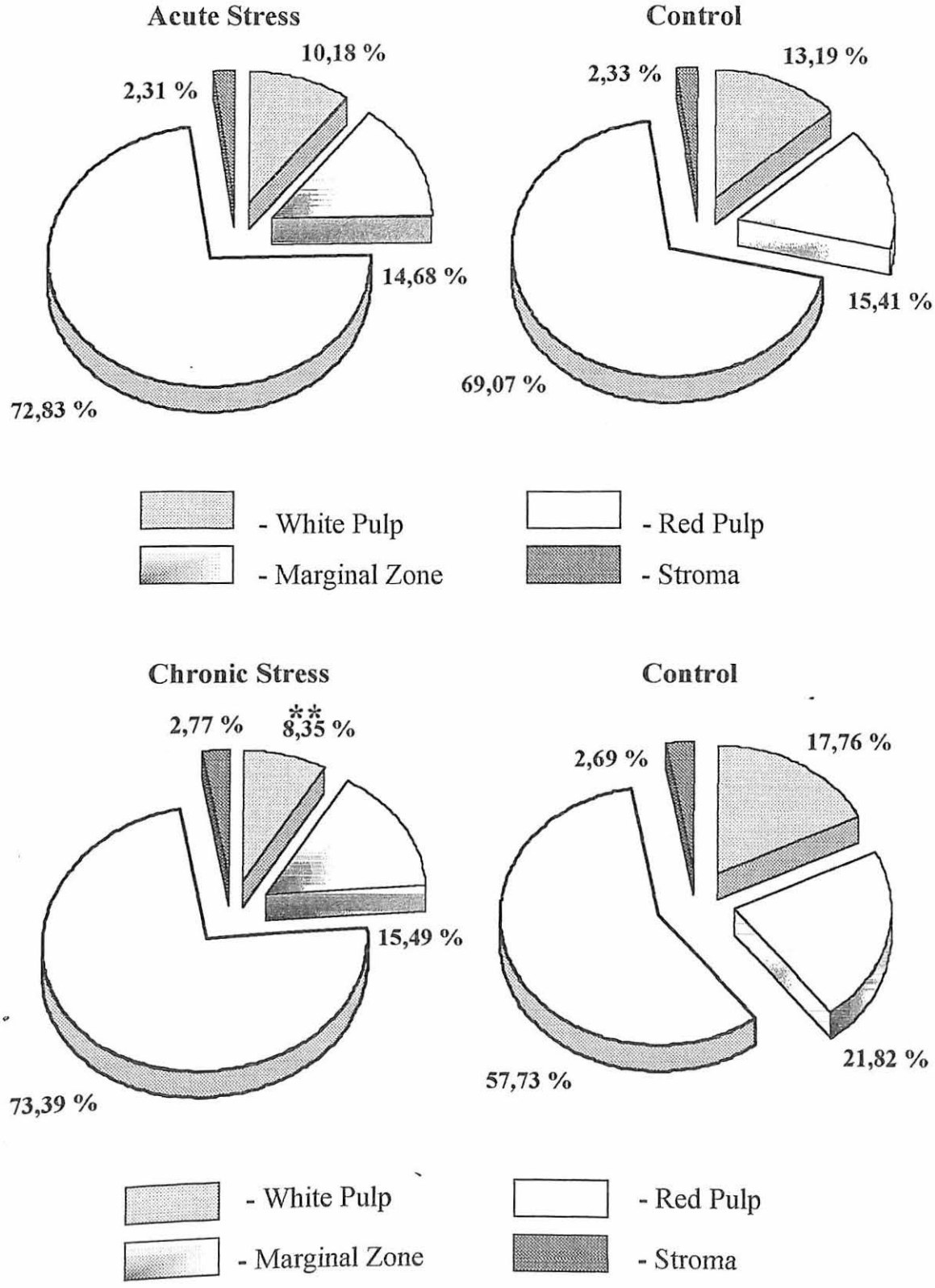
Results. The quantitative age-related characteristics of the splenic compartments: white pulp, marginal zone, red pulp and fibrous stoma (capsule and trabeculae) is presented in the diagrams 1-4. As it is seen on the diagram acute stress affected the structure of preweaning animals' spleen by reduction of the volume density of the white pulp and marginal zone, though the difference was not significant. This may be explained by a relatively refractive condition of the immune system to the corticosteroids of the rats

Diagram 1. **Volume Density of the Splenic Compartments in the Preweaning Rats under Acute and Chronic Stress**



* - - $p<0,05$ when compared against corresponding control

Diagram 2. **Volume Density of the Splenic Compartments in the Weaning Rats under Acute and Chronic Stress**



** - - $p<0.01$ when compared against corresponding control

Diagram 3. **Volume Density of the Splenic Compartments in the Infant Rats under Acute and Chronic Stress**

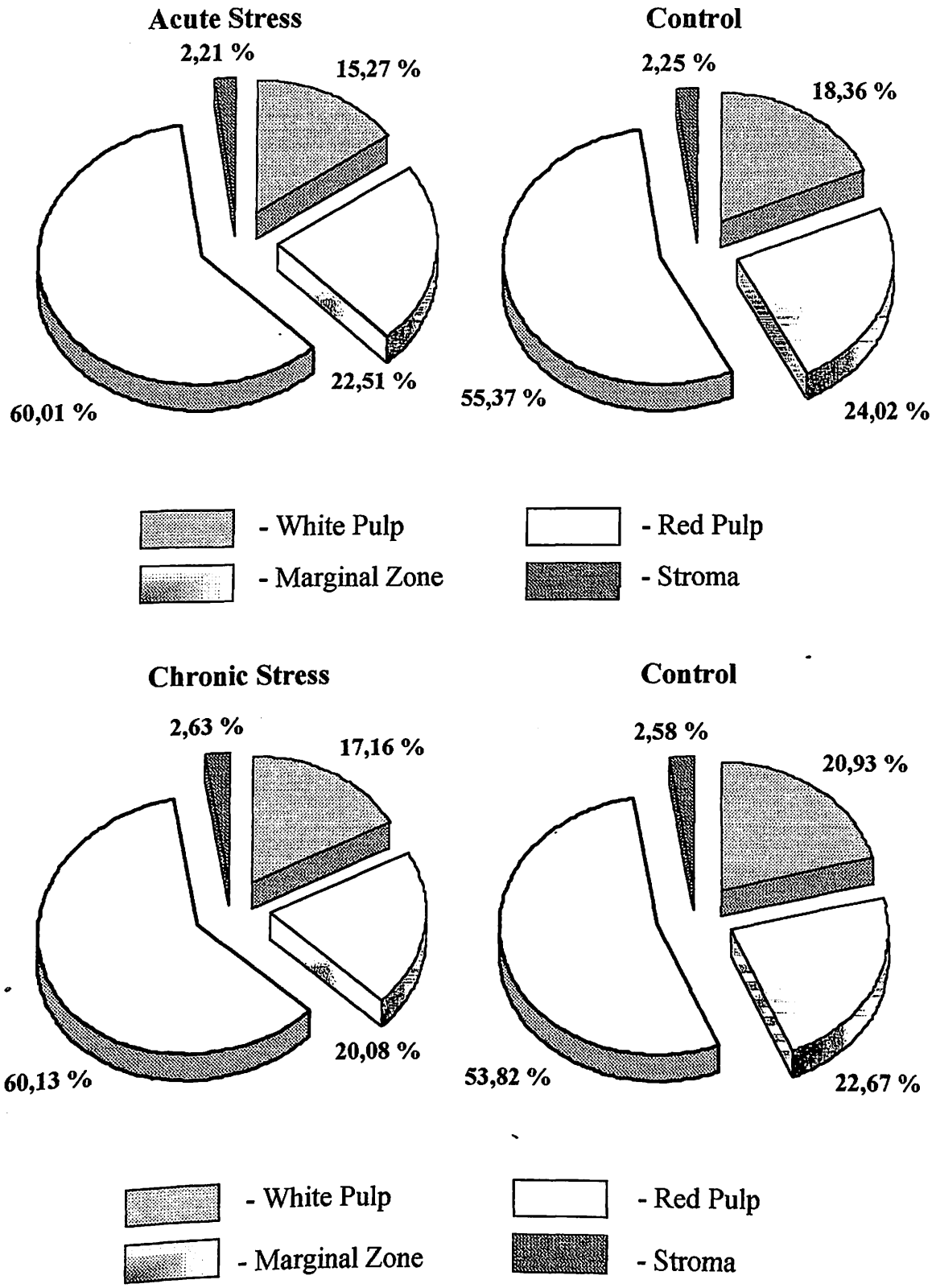
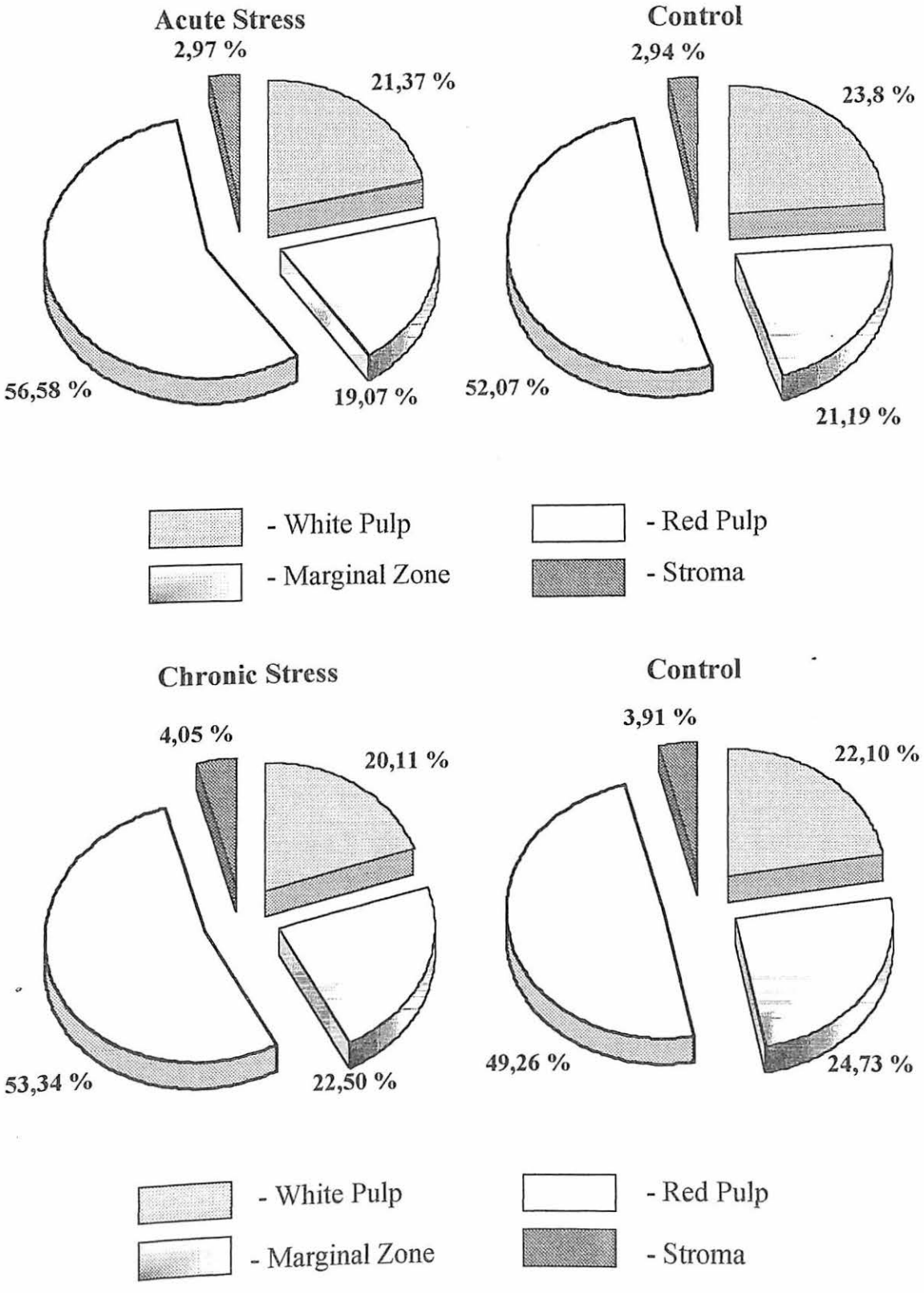


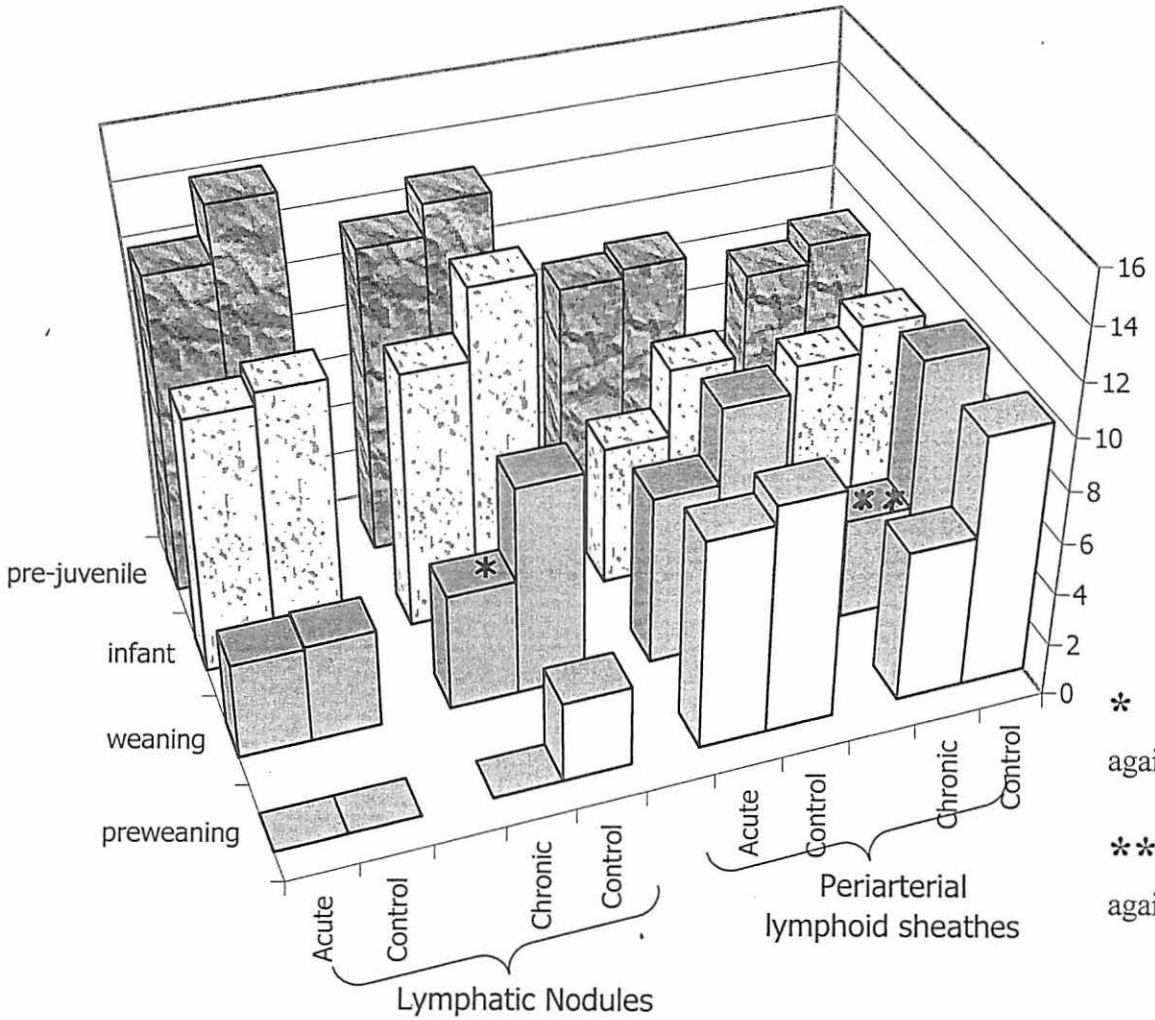
Diagram 4. Volume Density of the Splenic Compartments in the Pre-Juvenile Rats under Acute and Chronic Stress



between days 3 to 13 of early postnatal life (A.F. El Fouhil et al., 1995). Chronic stress applied at this age had much more prominent influence on the immune organs of the rat pups: by the end of the last restraint session on the 21 day of life both white pulp and marginal zone were significantly reduced (diagram 1, $p<0.05$). Weaning rats when exposed to both acute and chronic restraint stress turned out to be even more vulnerable than the preweaning rat pups in terms of the immunosuppresion: in chronic stress the volume of the white pulp was greatly reduced, the difference between experimental and control animals values being highly significant ($p<0.01$). In the infant and pre-juvenile animals the tendency of reduction of the white pulp and the marginal zone remained obvious though difference in their volume densities between experimental and control animals were not significant.

At the age of 2 weeks white pulp is represented by periarterial lymphoid sheaths (PALM) surrounded by a well-developed marginal zone, while B-cell compartment is absent. Immunohistochemical staining of spleen for B-cells at this age was also negative, while staining for CD90 revealed the presence of recent thymic immigrants in the splenic T-zones. Under normal conditions by the end of the 3rd week of early prenatal development primary lymphoid follicles appear in the white pulp while secondary follicles may not be identified before the 4th week of postnatal life. In preweaning animals under chronic stress exposure the lymphoid follicles failed develop by weaning period (Diagram 5). In the weaning rats exposed to chronic stress the primary lymphoid follicles having appeared by the time of the first session of stress exposure failed to develop further and remained immature (photo 1,3). As it is shown on the diagram 5, in the weaning rats exposed to the chronic stress the volume density of the lymphoid nodules was significantly reduced against control (4.45% against

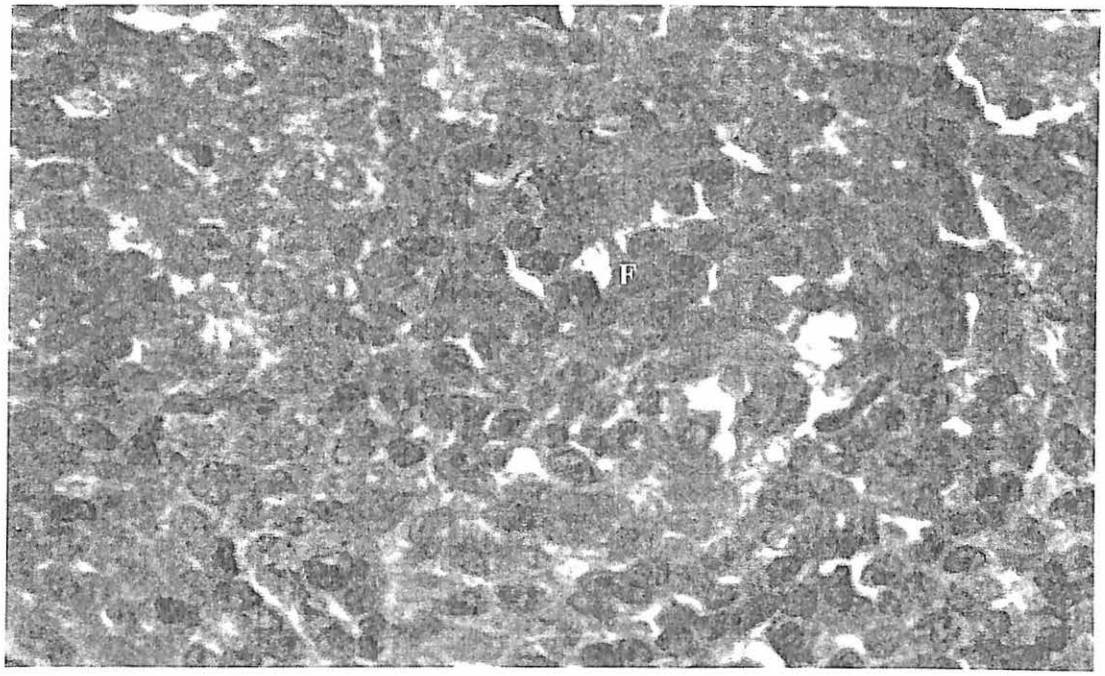
Diagram 5. Volume Density of the Splenic White Pulp Sub-Compartments in the Growing rats under Acute And Chronic Stress



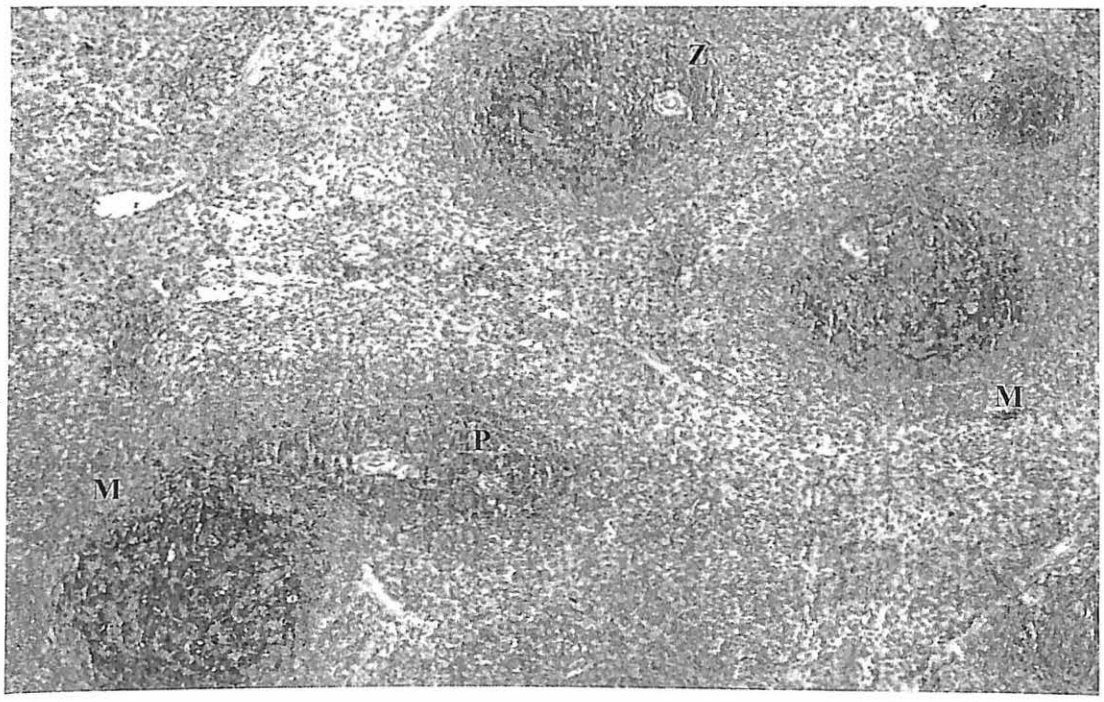
Legend:

* - $p<0.05$ when compared against corresponding control

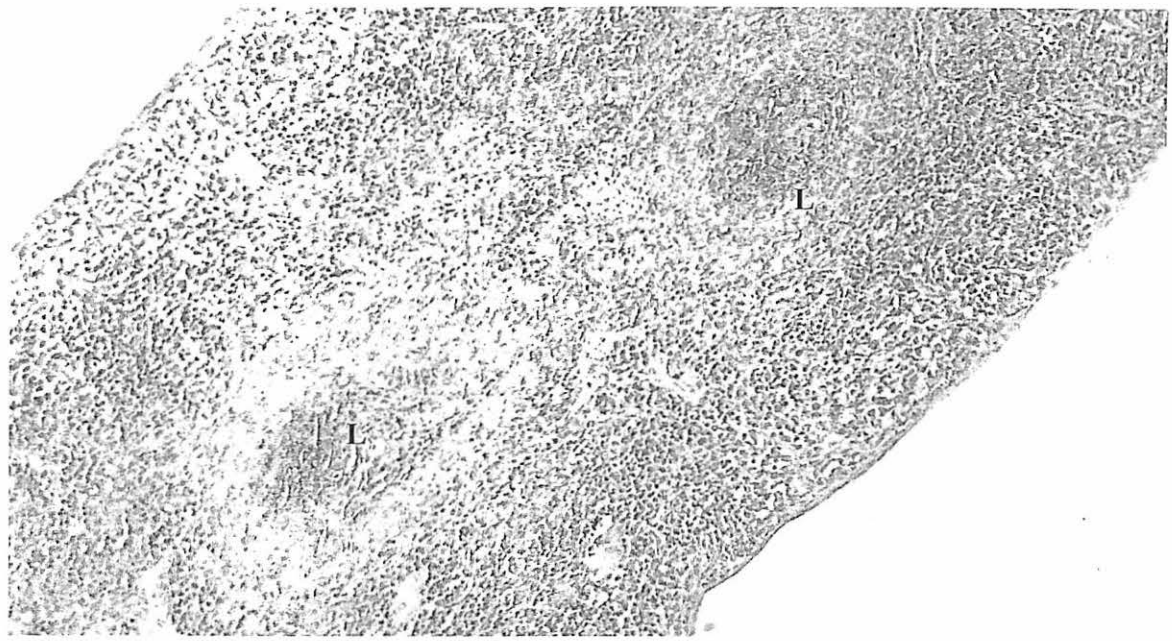
** - $p<0.01$ when compared against corresponding control



Picture 1. Microphotograph of the spleen of the control 21-day old Sprague-Dawley albino rat. Immunohistochemical staining for S100-protein + haemotoxylin. Immature lymphoid follicle (F) with few weakly S-100-positive stromal cells. Initial magnitude x400.



Picture 2. Microphotograph of the spleen of the control 30-day old Sprague-Dawley albino rat. Immunohistochemical staining for S100-protein + haemotoxylin. Three large lymphoid follicles with highly S-100-positive stroma of dendritic cells are displayed. Periarterial zones (Z) of the two of them are S-100-negative. Periarterial lymphoid sheath (P) and marginal zones (M) are also S-100-negative. Initial magnitude x40.



Picture 3. Microphotograph of the spleen of the 28-day old Sprague-Dawley albino rat after 7 daily sessions of restraint stress. Immunohistochemical staining for S100-protein + haemotoxylin. Lymphoid nodules (L) are rare, S100+cells in their stroma are sparse. Initial magnification x100

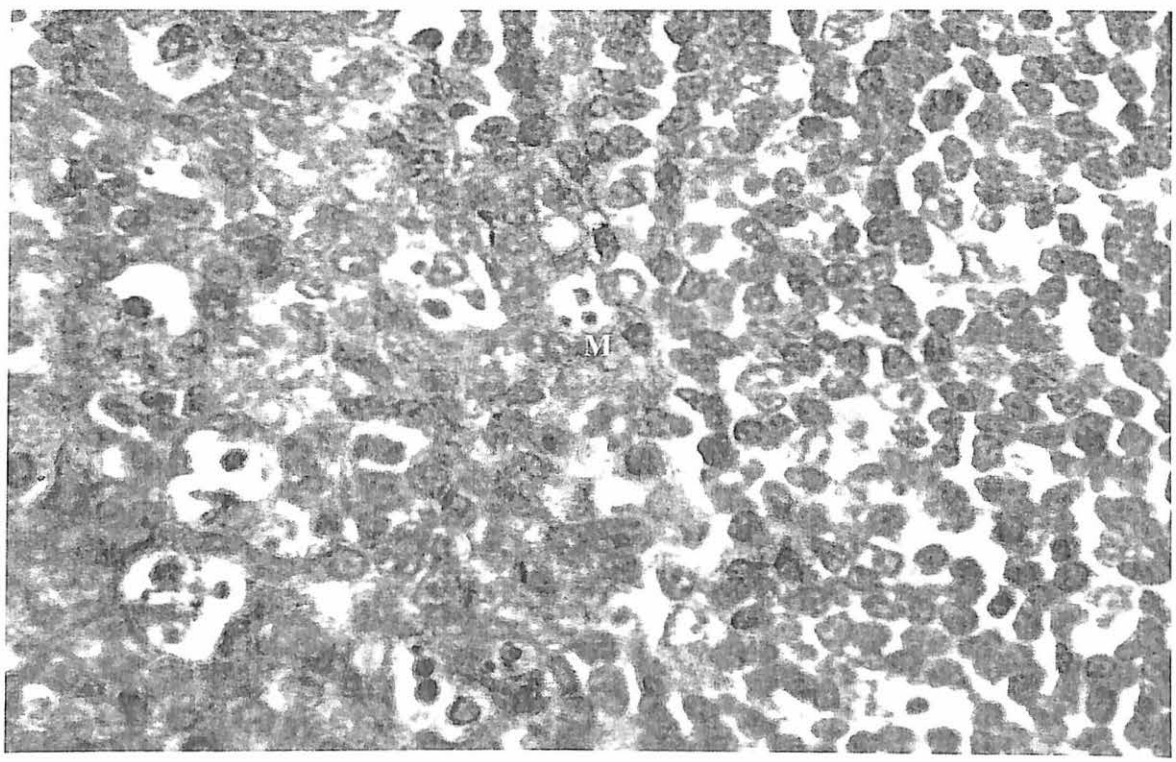


Photo 4. Photomicrograph of the spleen of the 30-day old Sprague-Dawley albino rat after 5 hours of restraint stress. Immunohistochemical staining for S100-protein + haemotoxylin. S-100-positive cells of dendritic appearance are observed in the lymphatic follicle. Multiple tingible-body macrophages (M) and free apoptotic bodies are present in the follicle. Initial magnification x400

8.06% accordingly, $p<0.05$) while the volume density of periarterial lymphoid sheaths was highly significantly decreased (3.9% against 9.7%, $p<0.01$). By the end of the 1st month of life the lymphoid follicles in the control rats reached rather big size with a broad band of surrounding marginal zone, occasional secondary lymphoid follicles might be distinguished between them. Under acute stress exposure many lymphocytes in the lymphoid follicles died by apoptosis with many apoptotic bodies and tingible-body macrophages identifiable in their mantle zone (Photo 2,4). The apoptotic bodies and tingible-body macrophages in the T-zones of the splenic white pulp were less common. Thus, both acute and chronic stress affected the structure of the largest secondary immune organ - spleen in terms of both T- and B-zoned of the white pulp including their marginal zone. The weaning rats' spleen proved to be the most sensitive to the stress exposure responding by dramatic reduction of the white pulp which is a convincing evidence of immunosuppression (C. Gopinath et al., 1996).

More details regarding immunomodulation in the growing body-under stress conditions were expected from the image analysis of the subcompartments of the splenic white pulp, such as the periarterial lymphoid sheaths and lymphoid nodules. The results are shown in the tables 1-8. We have evaluated several parameters characterizing both subcompartments, such as cross-section area, perimeter, maximal and minimal diameter, Ferret coefficient, volume density and numeric density. Regarding the periarterial lymphoid sheaths the two parameters proved to be the most informative, namely area of the cross section and width of the lymphocyte layer. Both parameter were continuously increasing throughout the prepubertal period of life. Immunohistochemical staining revealed the presence of the concentric layers of CD8+ and CD45RC+ lymphocytes around the arterioles as early as

Table 1

Quantitative Characteristics of the PALS in the Preweaning Rats under Acute and Chronic stress Conditions (M+/-m).

Group / Parameter	Acute Stress	Control	Chronic Stress	Control
Width of the Layer of Lymphocytes, mm	0.0201±0.002	0.0212±0.004	0.0197±0.003 *	0.028±0.002
Cross- section area, mm^2	0.0028±0.0004	0.0032±0.0004	0.0033±0.0005 **	0.0056±0.0006

Table 2

Quantitative Characteristics of the PALS in the Weaning Rats under Acute and Chronic stress Conditions (M+/-m).

Group / Parameter	Acute Stress	Control	Chronic Stress	Control
Width of the Layer of Lymphocytes, mm	0.0256±0.004	0.0284±0.003	0.0228±0.002 *	0.0313±0.004
Cross- section area, mm^2	0.0042±0.0004	0.0056±0.0006	0.0038±0.0004 **	0.0060±0.0008

Table 3

Quantitative Characteristics of the PALS in the Infant Rats under Acute and Chronic stress Conditions (M+/-m).

Group / Parameter	Acute Stress	Control	Chronic Stress	Control
Width of the Layer of Lymphocytes, mm	0.0301±0.003	0.0326±0.004	0.0290±0.002	0.0343±0.003
Cross-section area, mm^2	0.0055±0.0006	0.0063±0.0006	0.0052±0.0005	0.0066±0.0007

Table 4

Quantitative Characteristics of the PALS in the Pre-Juvenile Rats under Acute and Chronic stress Conditions (M+/-m).

Group / Parameter	Acute Stress	Control	Chronic Stress	Control
Width of the Layer of Lymphocytes, mm	0.0319±0.003	0.0357±0.004	0.0321±0.005	0.0349±0.004
Cross- section, mm^2	0.0061±0.0007	0.0071±0.0008	0.0052±0.0007	0.0062±0.0008

- * - $p<0.05$ when compared to the corresponding control
- ** - $p<0.01$ when compared to the corresponding control

Table 5

Quantitative Characteristics of the Lymphoid Follicles in the Preweaning Rats under Acute and Chronic stress Conditions (M+/-m).

Parameter \ Group	Acute Stress	Control	Chronic Stress	Control
Area of cross section, mm^2	0	0	0***	0.0075±0.001
Numeric Density mm^{-2}	0	0	0***	4.8±0.45

Table 6

Quantitative Characteristics of the Lymphoid Follicles in the Weaning Rats under Acute and Chronic stress Conditions (M+/-m).

Parameter \ Group	Acute Stress	Control	Chronic Stress	Control
Area of cross section, mm^2	0.0075±0.0008	0.0077±0.0008	0.0079±0.0008	0.0102±0.0011
Numeric Density mm^{-2}	4.69±0.49	4.81±0.57	3.95±0.43*	6.06±0.66

Table 7

Quantitative Characteristics of the Lymphoid Follicles in the Infant Rats under Acute and Chronic stress Conditions (M+/-m).

Parameter \ Group	Acute Stress	Control	Chronic Stress	Control
Area of cross section (primary follicles), mm^2	0.0114±0.004	0.0116±0.003	0.0135±0.005	0.0172±0.002
Area of cross section (secondary follicles), mm^2	0.0270±0.003	0.0273±0.004	0.0294±0.006 *	0.0383±0.005
Numeric Density mm^{-2}	5.11±0.53	6.09±0.58	3.87±0.39	4.01±0.51

- * - $p<0.05$ when compared to the corresponding control
- *** - $p<0.001$ when compared to the corresponding control

Table 8

Quantitative Characteristics of the Lymphoid Follicles in the Prejuvenile Rats under Acute and Chronic stress Conditions (M+/-m).

Group / Parameter	Acute Stress	Control	Chronic Stress	Control
Area of cross section (primary follicles), mm^2	0.0188+/-0.004	0.0191+/-0.005	0.0249+/-0.005	0.0286+/-0.004
Area of cross section (secondary follicles), mm^2	0.0270+/-0.003	0.0273+/-0.004	0.0530+/-0.006	0.0581+/-0.007
Numeric Density mm^{-2}	3.75+/-0.41	3.85+/-0.31	3.17+/-0.48	3.50+/-0.62

in one day old rat pups with CD45RC+ cells locating in the outer layers of the developing periarterial lymphoid sheaths. Image analysis has displayed the significantly decreased size of the periarterial sheaths in preweaning and weaning rat pups under chronic stress conditions compared to their age-matched control. The reduction of these parameters was observed in the infant and pre-juvenile animals but the difference of values was not significant. Regarding lymphoid follicles, the most informative parameters to describe the dynamics of their development in prepubertal rats were numeric density and cross-section area (tables 5-8). It has been shown that these parameters were affected by chronic stress exposure in the preweaning, weaning and infant rats with the changes of the values in the pre-juvenile rats being not significant. These data enable us to conclude that T- and B-zones are differentially affected by stress exposure in different age groups: considerable T-zone suppression under chronic stress conditions takes place before infancy while B-zone immunosuppression continues during infancy, particularly in terms of reduction of the secondary follicles.

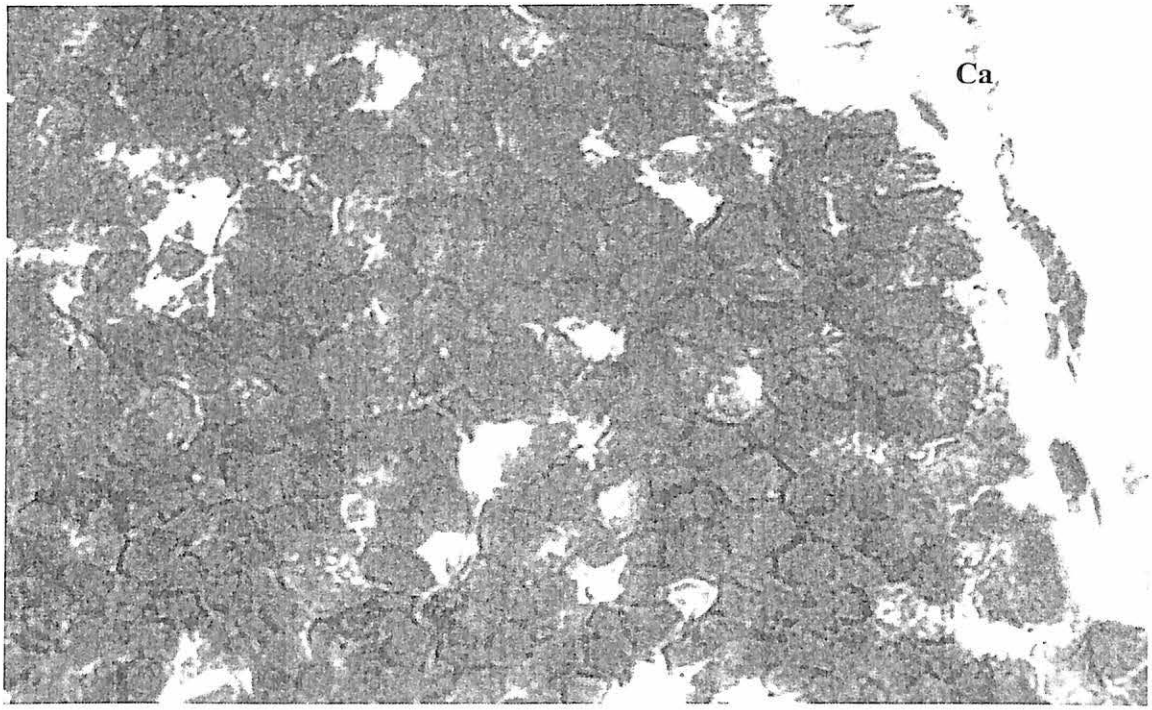
Immunohistochemical staining revealed considerable changes of the phenotype of the immunocytes both under acute and chronic stress. It has been demonstrated that under acute stress in the spleen of the weaning rats of the apoptosis of the immunocytes was mainly confined to the lymphatic nodules particularly to their germinal centers where apoptotic bodies and positive staining for caspase-3 (enzyme of the early stages of apoptosis) were detected. Single immunoreactive cells were discovered in the T-zones (periarterial lymphoid sheathes and periarterial zones of the lymphatic follicles) as well as in the marginal zone of the lymphatic follicles and the red pulp. In the infant animals incidence of spleen immunocyte apoptosis in the aforesaid zones proved to be even higher in spite of a pronounced

macrophageal reaction. These findings enable us to conclude that apoptosis plays an essential role in hypocellularity of the immune organs under acute restraint stress conditions on the contrary to the data of some other investigators who believe that depletion of the lymphocytes from the lymphoid organs is mainly responsible for their hypoplasia due to the surge of the concentration of corticosteroid in blood during stress reaction. Preliminary estimation of the apoptosis rate in the lymphoid follicles of spleen of the metyrapone-treated animals revealed the its reduction under acute stress conditions against non-treated animals, but these results need further evaluation.

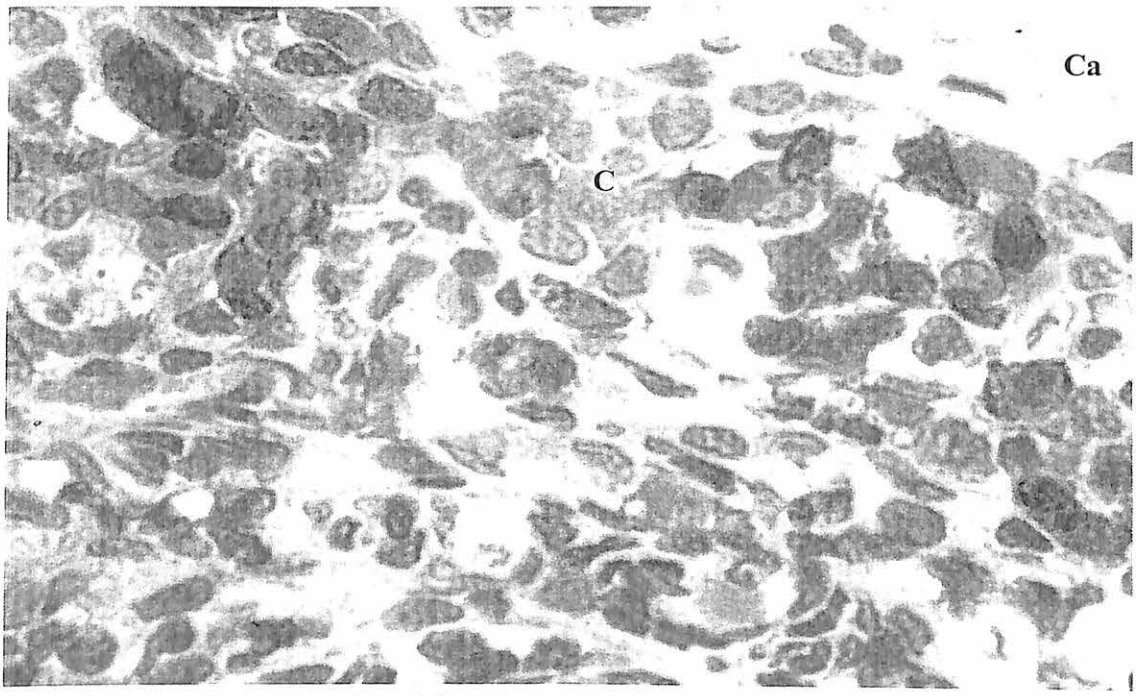
Immunohistochemical staining for CD45RC and CD90 demonstrated that under chronic stress conditions CD45RC-CD90+ cells disappear from the periarterial lymphoid sheaths demonstrating a reduced influx of the recent thymic immigrants. This finding correlated with the observation made in immunohistochemical evaluation of thymus under repeated exposures to the stressful factor: the amount of CD3+ and CD8+ lymphocytes is drastically reduced both in the cortex (for CD8-immunoreactive cells) and medulla (CD3-immunoreactive cells) (photo 5,6). At the same time the amount of CD8+ cells may increase in the periarterial lymphoid sheaths of the growing rats starting from the weaning period thus demonstrating different aspects of immunomodulation under acute and chronic stress conditions. Staining for CD45RC and pan-B-Cell demonstrated prominent reduction in the amount of immunoreactive cells in the marginal zone and lymphoid follicles with B-compartments of the weaning rats being most vulnerable in terms of immunomodulation.

Discussion. In the present investigation for the first time a detailed quantitative microscopic and immunohistochemical characteristics of the

immune organs (spleen and thymus) of the growing body under stress conditions was provided. Until recently only sparse information was available on the immunomodulation in the growing body under stress conditions, these data were obtained mainly from the immunological investigation of cell cultures of cell mixtures (M.Odio et al., 1987; E.Ortega et al., 1994; K.Takahashi et al., 1997; Dominguez-Gerpe L., 2001). At the same time it is widely known that immune organs are highly compartmentalized and complete information regarding immunomodulation may be obtained only from the separate analysis of every compartment, sub-compartment and domain of the lymphoid organs (C.Gopinath, 1996). Under stress conditions the borders between the compartments and subcompartments of the immune organs often become indistinct and adequate evaluation of the immunomodulation may be performed only using immunohistochemical analysis of the cellular phenotype. On the contrary to some other investigators (S.Zalcman et al., 1993; R.L.Spencer et al., 1996) we discovered using image analysis and immunohistochemical staining of the lymphoid organs that stress, both acute and chronic, adversely affects both T- and B-zones of the immune organ resulting in suppressed cellular and humoral immunity. Certain increase of the CD8+cells in spleen after a single stress session may not be considered as enhancement of the immune function as for the effective immune response certain balance between CD4+ and CD8+ lymphocytes is required and it is unlikely that the immunocytes of any other phenotype increase in number in any of the immune organs under either acute or chronic stress conditions. On the contrary to some investigators which believe that cell depletion under the increased concentration of the corticosteroid hormones in blood in stress is responsible for the hypoplasia of the immune organ, we demonstrated using



Picture 5. Microphotograph of the the thymic cortex of the 21-day old control rat. Immunohistochemical staining for CD8, counterstaining with hematoxylin. The majority of lymphocytes of the subcapsular region are immunoreactive. Ca – capsule. Initial magnification x400



Picture 6. Microphotograph of the thymus of the 21-day old rat exposed to stress starting from the preweaning period (14th day of life). Immunohistochemical staining for CD8, counterstaining with hematoxylin. Few lymphocytes in the subcapsular region of the cortex (C) are immunoreactive. Ca – capsule. Initial magnification x400.

immunohistochemical staining for caspase-3 an important role of splenic and thymic lymphocyte apoptosis in the immunosuppression of the growing animals under stress conditions. More information on the different manifestations of the immune response deviations in the growing body may be obtained from the quantitative immunohistochemical examination of the lymphoid organs which we intend to commence upon the completion of the present project.

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