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Stereological study of splenic tissue compartments in FK506-treated rats

N.M. Milićević and Ž. Milićević

Institute of Histology and Embryology, Faculty of Medicine, University of Beograd, Beograd, Yugoslavia

Summary. Model-based stereology was used to study the changes of splenic structure and proportions of splenic tissue compartments in FK506-treated male Wistar rats (0.1 mg/kg three times a week for 14 days). The point-counting method was utilized to study the volume densities of the following tissue compartments: red pulp; white pulp (this compartment was divided in two subcompartments: follicles and periarteriolar lymphocyte sheath); marginal zone; and connective tissue. The following stereological parameters of lymphoid follicles were also determined: areal numerical density (the number of follicles per mm^2 of tissue section); the numerical density (number of follicles per mm^3 of tissue); and the mean follicle diameter. After FK506 treatment a significant reduction of volume density of white pulp was observed, which was due to the very prominent decline in the amount of periarteriolar lymphocyte sheath, whereby the cellular density of this tissue compartment was decreased. In the marginal zone of FK506-treated spleens the presence of numerous, large, densely packed lymphoblastic cells of very uniform appearance was registered. These observations are in very good keeping with the changes of splenic morphology registered in cyclosporin A-treated rats, which further documents the parallelism in actions between these immunosuppressive agents.

Key words: Spleen, Splenic tissue compartments, FK506, Immunomodulation, Stereology

Introduction

FK506 is an immunosuppressive drug isolated from the fermentation broth of *Streptomyces tsukubaensis* and belongs to the macrolide family (Kino et al., 1987). Although structurally different, it shows many similarities in its immunosuppressive activity to cyclosporin A, whereby the central events are inhibition of interleukin-2 and other cytokines production by helper T-lymphocytes and blocking in generation of cytotoxic T-lymphocytes, albeit at 100 times lower concentrations (Sawada et al., 1987). Thus, FK506 strongly suppresses graft rejection and development of experimentally-induced autoimmune diseases (Ochiai et al., 1987; Takabayashi et al., 1989). Therefore, FK506 is emerging as a drug with great therapeutic potential in human medicine (Thomson, 1989) and as a valuable addition to the already established immunosuppressant cyclosporin A.

It has been shown that the inhibitory action of FK506 on the immune activity is accompanied by blocking in thymocyte maturation and structural alterations within the thymus, that is, the prominent reduction of thymic medulla (Pugh-Humphreys et al., 1990; Takai et al., 1990). However, the data on structural changes in other lymphatic organs, particularly in the spleen, after FK506-treatment are lacking in the literature.

The spleen is the largest peripheral lymphatic organ which, acting as the sole blood filter of the organism, performs the integrative and defensive functions that cannot be accomplished by other organs of the immune system. It has a delicate microarchitecture, with finely organized vascular network. Various compartments of the splenic tissue, i.e., red pulp, marginal zone and white pulp (with its subcompartments), are populated with distinct lymphoid and nonlymphoid cells, which have different functional roles (Pabst and Westermann, 1991). After organ transplantation the spleen undergoes prominent structural changes affecting all of its tissue compartments (Baldwin et al., 1979; Wolf, 1987). Considering this and the lack of data on the effects of FK506 on the structure of the spleen, we believed that it would be interesting to perform a detailed morphological and morphometrical study of all splenic tissue compartments after the application of this agent.

Materials and methods

Animals

Six-week-old, male Wistar rats (obtained from ICN-Galenika Farm, Beograd), with body weights of 140-160 g at the beginning of the experiment, were used. The animals were given standard laboratory chow and tap

Offprint requests to: Prof. Dr. Novica Milićević, Dim. Tucovića 83, 11000 Beograd, Yugoslavia

water *ad libitum*.

Experimental protocol

Six animals received a daily dose of 0.1 mg of FK506 per kg of body weight (a kind gift of Fujisawa Pharmaceutical Co., Osaka, Japan) dissolved in 70% ethanol. The rats were given the drug by intraperitoneal injection three times a week in the volume of 0.2 ml of the solvent for two weeks. Two days after the last injection the rats were weighed and sacrificed by exsanguination in ether anaesthesia. Five rats from the same litter served as control.

Preparation of tissue for microscopy

The spleen was removed, weighed and slices of splenic tissue were fixed in 10% neutral-buffered formalin or in Bouin's solution. The tissues were routinely processed and embedded in Paraplast. From each block, several 6 µm-thick sections were taken at 3 different levels of tissue 200 µm apart. Thereafter, the sections were routinely stained with hematoxylin and eosin.

Spleen Histomorphometry

Volume densities of the rat spleen tissue compartments

The point-counting method was used. The volume density of a tissue compartment i (V_{vi}) was calculated from the equation: $V_{vi}=P_i/P_t$, where P_i=number of points falling in the tissue compartment i, and P_t=total number of points counted on the reference tissue (Weibel, 1979). The following tissue compartments were studied (Fig. 1): 1) red pulp (V_{vrp}); 2) marginal zone (V_{mz}); 3) white pulp (V_{vwp}) - this tissue compartment was divided into two subcompartments; 3a) follicles (V_{vfoll}) and 3b) periarteriolar lymphocyte sheath (PALS - V_{vPALS}); and 4) connective tissue of capsule and trabeculas (V_{ct}). All determinations were performed at a magnification of x160 using lattice with 36 points (B 36). Three sections per spleen were taken from different tissue levels, 200 µm apart and utilized for the analysis using the systematic field sampling technique.

Weights (in mg) of spleen tissue compartments were calculated by multiplying the corresponding volume density by the spleen weight.

Stereological parameters of the rat spleen follicles

The following parameters were calculated: 1) areal numerical density (the number of follicles per mm^2 of tissue section - N_{Af}); 2) the numerical density (number of follicles per mm^3 of tissue - N_{Vf}) and 3) the mean follicle diameter ($\bar{D}$). The numerical density of the follicles per 1 mm^3 of spleen tissue (N_{Vf}) was calculated from the following equation (Weibel, 1979):

$$N_{Vf} = {}^{1}\!/\!_{\beta} \times K \times \sqrt{{}^{N_{Af}^{3}}\!/\!_{V_{Vf}}}$$

where N_{Af}=number of follicles per mm^2 of spleen section; V_{Vf}=volume density of the follicles, (this was determined by the point-counting method on 3 spleen sections using a magnification of x160 and lattice B 36); ß=0.87 (shape factor of the follicles); K=1.06 (factor for the size distribution of the follicles; Weibel, 1979). The mean follicle diameter ($\bar{D}$) was calculated from the equation (Weibel, 1979):

$$\bar{D} = 2 \times \sqrt[3]{{}^{3}\!/\!_{4\pi} \times {}^{V_{Vf}}\!/\!_{N_{Vf}}}$$

which is based on the assumption that the follicles have a spherical shape.

The total number of the follicles in the spleen (N_{Tf}) was calculated from the following equation (Weibel, 1979): $N_{Tf}=W/s \times f_3 \times N_{Vf}$, where W=spleen weight; s=specific gravity of the spleen tissue (=1.27); f_3=factor for the tissue shrinkage in cubic dimensions (=0.59).

The mean values for each scoring procedure were determined and standard deviation calculated. Student's t-test was employed for comparing of the means.

Results

Control animals

The mean weight of spleen of the control rats was 480.0 mg. Red pulp, marginal zone and white pulp with its subcompartments were well developed and easily discernible in the control spleen (Fig. 1). The volume densities of tissue compartments of control spleens are presented in Table 1, whereas the absolute weights are shown in Table 2 and given in parenthesis in the text. The volume density of splenic red pulp and marginal zone was 54.6% (261.9 mg) and 16.8% (80.9 mg)

Table 1. Volume densities (%) of splenic tissue compartments in control and FK506-treated rats (means±SE).

PARAMETER	V_{vrp}	V_{vmz}	V_{vwp}	V_{vfoll}	V_{vPALS}	V_{vct}
Control rats	54.6±4.8	16.8±1.7	23.2±3.7	6.4±0.6	16.7±3.1	5.7±1.7
FK506-treated rats	57.3±4.1	23.6±3.2*	14.4±2.1*	8.7±1.4	5.6±1.2*	4.7±0.8

V_{vrp}: volume density of red pulp; V_{mz}: volume density of marginal zone; V_{vwp}: volume density of white pulp; V_{vfoll}: volume density of follicles; V_{VPALS}: volume density of periarteriolar lymphocyte sheath; V_{vct}: volume density of connective tissue; *: statistically significant difference.

respectively. The marginal zone was loosely populated with lymphoid cells of varying appearance, the majority of them were medium-size lymphocytes (Fig. 2). White pulp was 23.2% (108.0 mg), whereas follicles and PALS constituted 6.4% (30.9 mg) and 16.7% (80.2 mg) respectively. PALS contained densely packed small lymphocytes (Fig. 3). The proportion of connective tissue was 5.7% (27.0 mg). Various stereological parameters of the follicles of control rats are presented in Table 3. The number of follicles was 0.6. Areal and

Table 2. Weight (mg) of splenic tissue compartments in control and FK506-treated rats (means±SD).

PARAMETER	SPLEEN (total)	RED PULP	MARGINAL ZONE	WHITE PULP	FOLLICLES	PALS	CONNECTIVE TISSUE
Control rats	480.0±7.0	261.9±19.2	80.9±9.6	108.0±16.9	30.9±3.4	80.2±16.1	27.0±8.0
FK 506-treated rats	404.1±49.7	230.7±22.5	96.4±22.4	57.8±8.3*	34.4±6.0	22.4±3.7*	19.0±4.6

*: statistically significant difference ($p<0.001$).

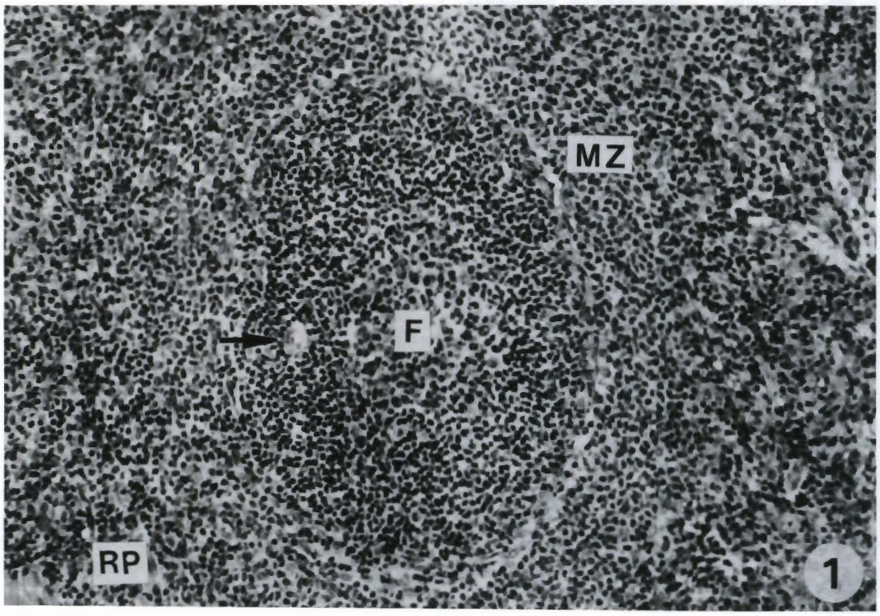

Fig. 1. Spleen of the control rat. Large secondary follicle (F). Well developed peri-arteriolar lymphocyte sheath with central artery (arrow) and marginal zone (MZ). RP: red pulp. Hematoxylin-eosin. x 125

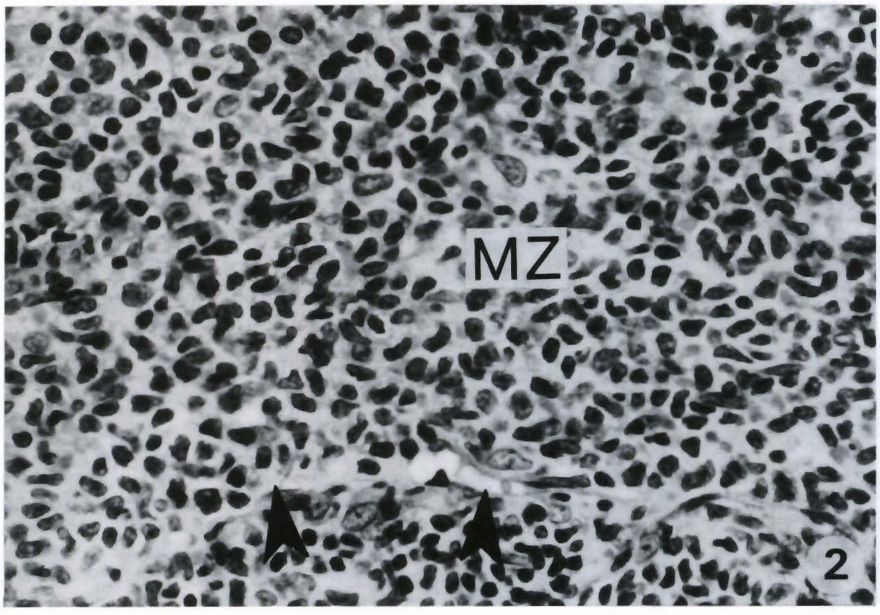

Fig. 2. Spleen of the control rat. Marginal zone (MZ) is populated with cells of varying size and appearance. Arrowheads: marginal sinus. Hematoxylin-eosin. x 350

numerical densities were 1.6 and 11.1 respectively. Total number of follicles in the control spleen was 839.0 and volume density of follicles was 0.06. Mean diameter of control follicles was 223.9 μm.

FK506-treated animals

The mean weight of the spleen of FK506-treated rats was 404.1 mg, which was lower than that in control

Table 3. Stereological parameters of splenic lymphoid follicles (means±SE) in control and FK506-treated rats.

PARAMETERS	N_{Af} (mm^{-2})	N_{vf}(mm^{-3})	N_{Tf}	V_{vfoll} (mm^3/mm^3)	$\bar{D}$ (μm)
Control rats	1.6±0.2	11.1±2.6	839.0±12.3	0.06±0.006	223.9±0.3
FK506-treated rats	1.6±0.2	8.9±2.1	551.7±96.9*	0.08±0.01	258.6±26.4

N_{Af}: number of follicles per mm^2 of section area; N_{vf}: number of follicles per mm^3 of tissue; N_{Tf}: total number of follicles in the spleen; V_{foll}: volume density of follicles; D: mean follicular diameter; *: statistically significant difference ($p<0.01$).

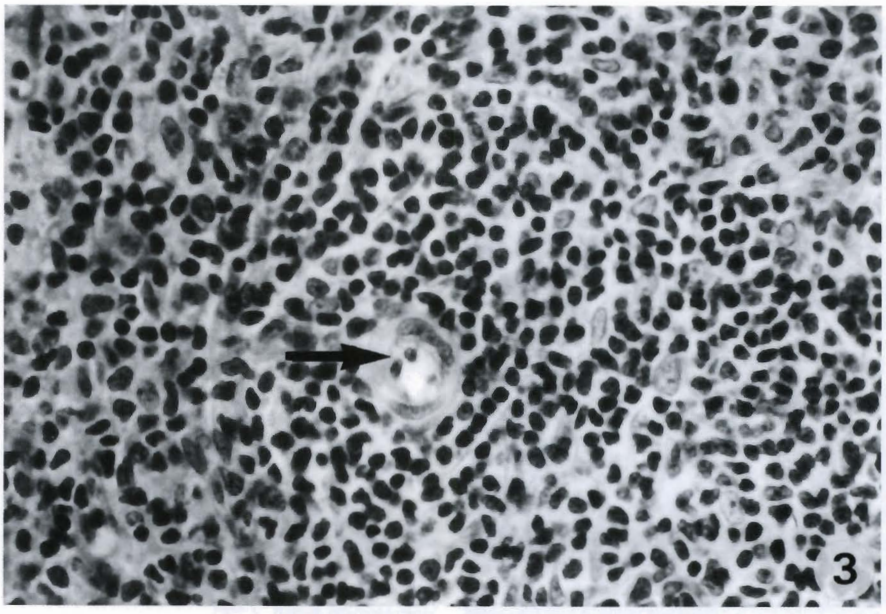

Fig. 3. Spleen of the control rat. Periarteriolar lymphocyte sheath is densely populated with small lymphocytes. Arrow: central artery. Hematoxylin-eosin. x 350

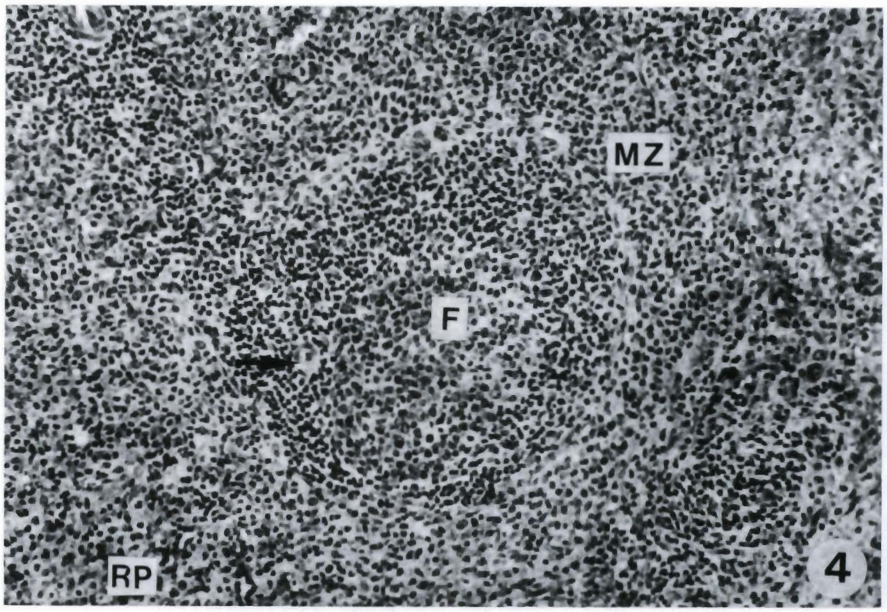

Fig. 4. Spleen of the FK506-treated rat. Diameter and cellular density of periarteriolar lymphocyte sheath are decreased. Marginal zone (MZ) is broadened with large-size lymphocytes of uniform appearance. Arrow: central artery. F: secondary follicle; RP: red pulp. Hematoxylin-eosin. x 125

animals. However, the difference was not statistically significant. Red pulp, marginal zone and white pulp were clearly recognizable. However, the organization (Fig. 4) and volumes of splenic tissue compartments were changed in animals treated with FK506. The volume densities of tissue compartments of FK506-treated spleens are presented in Table 1, whereas the absolute weights are shown in Table 2 and given in parenthesis in the text. The volume density of splenic red pulp was 57.3% (230.7 mg). The volume of marginal zone comprised 23.6%, which was significantly larger than control values ($p<0.05$). The absolute amount of marginal zone was 96.4 mg, which, however, was not significantly different from the control. The cellular composition of marginal zone was also altered. It was densely packed with large lymphoblastic cells of very uniform appearance (Fig. 5). White pulp was 14.4% (57.8 mg), which was significantly lower than control values ($p<0.01$). The follicles constituted 8.7% (34.4 mg). Volume density and weight of PALS were 5.6% and 22.4 mg respectively, which was significantly lower than control values ($p<0.001$; $p<0.001$). Moreover, the cellular density of PALS appeared decreased (Fig. 6). The remaining cells, however, did not morphologically

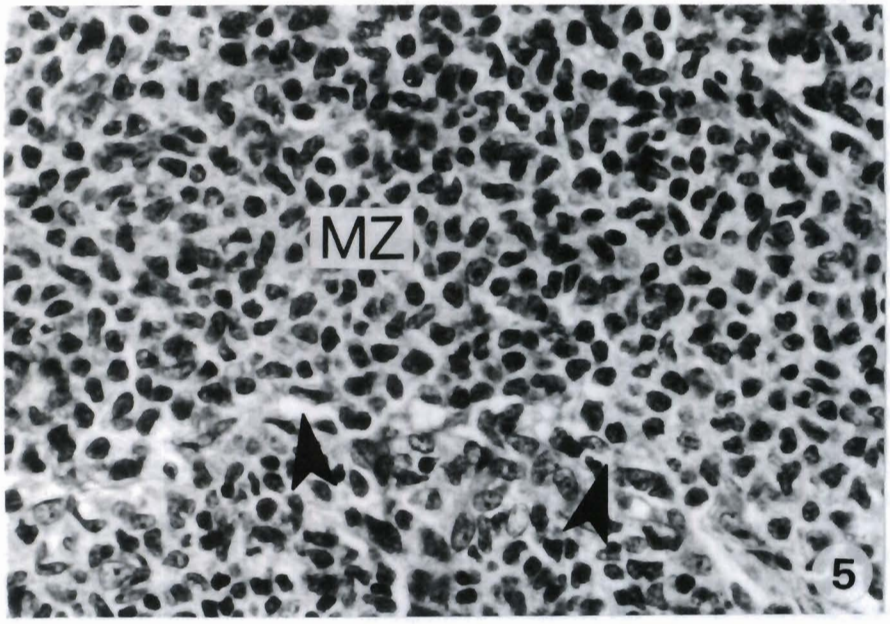

Fig. 5. Spleen of the FK506-treated rat. Marginal zone (MZ) is broadened and populated with numerous large-size lymphocytes of uniform appearance. Arrowheads: marginal sinus. Hematoxylin-eosin. x 350

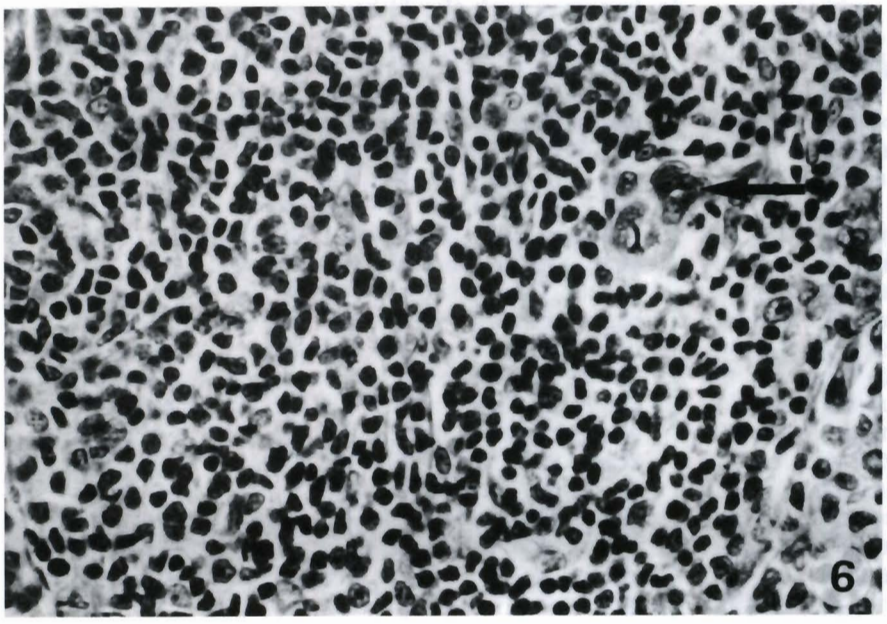

Fig. 6. Spleen of the FK506-treated rat. Decreased cellular density of periarteriolar lymphocyte sheath. Arrow: central artery. Hematoxylin-eosin. x 350

differ from cells residing in PALS of untreated rats. The proportion of connective tissue was 4.7% (19.0 mg). Various stereological parameters of the follicles of FK506-treated rats are presented in Table 3. The number of follicles was 0.5. Areal and numerical densities were 1.6 and 8.9 respectively. Total number of follicles in the spleen was 551.7, which was significantly less than in the control ($p<0.001$). Volume density of follicles was 0.08. Mean diameter of follicles was 258.6 μm.

Discussion

Our study is the first to investigate the morphogical changes in the spleen and alterations of splenic tissue compartments using a stereological approach after the application of FK506.

One of the most prominent changes which we observed in the spleens of FK506-treated rats, was the decreased volume density of splenic white pulp. Actually, the volume density of lymphoid follicles, as one of white pulp subcompartments, was not changed, but on the other hand the amount of PALS was significantly decreased. Thus, this significance was carried over to common value of white pulp. The decrease in volume density of PALS was accompanied with the decreased cellular density of this tissue compartment. It is known that FK506 is capable of impairing the activity of peripheral T-lymphocyte pool (Sawada et al., 1987), as well as blocking the maturation of thymocytes in the thymus (Takai et al., 1990). Thus, the decreased volume and cellular density of PALS after treatment with FK506 on the one hand may reflect the decreased production of mature T-lymphocytes in the thymus, which migrate to seed this tissue compartment of the spleen, and on the other hand are in agreement with the decreased activity of T-cells registered in functional assays (Sawada et al., 1987).

Recently, we have published the first complete stereological account on all tissue compartments of the human spleen (Milićević et al., 1996). Considering the species differences, the data on the volumes of tissue compartments of the rat spleen, as presented in this study, are comparable to those obtained earlier in human spleen (Milićević et al., 1996).

The observations regarding splenic marginal zone are also very interesting. The volume density of this tissue compartment was increased in comparison with the control. However, when calculated into the absolute amount the significance of this increase disappeared. But, in addition to these morphometrical changes, the alteration of cellular composition of marginal zone after FK506 application strongly attracts attention. In contrast to control spleens, which were populated with loosely arranged lymphoid cells of varying morphology (in respect with size, nuclear aspect, etc), marginal zone of FK506-treated spleens was densely packed with large, lymphoblastic cells of very uniform appearance. The nature and origin of cells, which appear in the marginal zone after application of either cyclosporin A (Hattori et al., 1987; Armas et al., 1989) or FK506 (this study) is unknown. The marginal zone of the intact spleen is populated with distinct lymphoid and nonlymphoid cells (Kraal, 1992), which are involved in the specific functional role of the spleen. This organ, in contrast to other lymphatic organs of the organism, has the unique potential to mount the primary immune response against encapsulated bacteria (so-called thymus-independent type 2 response). To fulfill this task the presence of an unimpaired marginal zone is crucial (Timens, 1991), because the marginal zone cells have the unique ability to bind polysaccharides from the bacterial capsule (Peset Llopis et al., 1996). Considering that the T-dependent immune response is suppressed in FK506-treated rats, it could be possible that the changes of the marginal zone in these animals are induced by increased penetration of bacteria from the gastrointestinal tract, which could elicit the response and transformation of marginal zone cells. Similar reactive expansion of marginal zone is also observed in the immunocompromised patients with B-cell lymphomas of gastrointestinal mucosa-associated lymphoid tissue (Harris et al., 1996). On the other hand, considering that both cyclosporin A and FK506 through the intracellular pathways interfere with expression of several lymphocyte genes (Kunz and Hall, 1993) the direct effects of these agents on marginal zone cells cannot be excluded. Thus, the described changes in the cellular composition of marginal zone after the application of both cyclosporin A and FK506 warrant further attention.

As there are no data in the literature on splenic morphology after application of FK506, our data may be only compared with the results of authors who studied the effects of cyclosporin A on the immune system. Both of our above mentioned observations are in very good keeping with the changes of splenic morphology registered after the application of cyclosporin A. Namely, the most striking alterations of splenic structure induced by this immunomodulatory agent were the marked decrease in size and lymphocyte depletion of PALS, as well as infiltration of marginal zone with large-size lymphocytes (Baldwin et al., 1981; Blair et al., 1982; Hattori et al., 1987). Armas et al. (1989) obtained similar results using histomorphometric methods to study the structure and tissue compartments of the rat spleen after treatment with cyclosporin A. They registered the decrease in size of white pulp, which was due to the reduction of PALS, whereby the cellular density of the latter was prominently decreased. They also noticed the appearance of large, pyroninophilic, lymphoblastic cells in the marginal zone. All this further documents the parallelism in actions between cyclosporin A and FK506 and shows that both of these agents not only affect the peripheral lymphocyte pool and the thymus in a similar manner, but the peripheral lymphatic organs as well.

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