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Stereologic Analysis of Tissue Compartments of Gunshot-injured and Blunt-injured Spleen

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Abstract The spleen is composed of several tissue compartments and the respective histoquantitative data are essential for complete understanding of immune or pathological processes in this organ. The aim of our study was to determine and compare the stereologic parameters of all tissue compartments of the gunshot-injured and blunt-injured human spleen. The model-based stereology with point-counting method was utilized to study the volume densities of red pulp, perifollicular zone, marginal zone, white pulp (follicles and periarteriolar lymphoid sheath), and connective tissue. The areal numerical density (the number of follicles per mm^2 of tissue section), the numerical density (the number of follicles per mm^3 of tissue) of lymphoid follicles and the mean follicle diameter were also determined. Our study provides stereological parameters for all tissue compartments of the human spleen. No morphometric differences were registered between tissue compartments of the blunt-injured and gunshot-injured spleen. As the gunshot-injured spleen was taken as presumably unstimulated in immunological regard, our results suggest that both gunshot-injured and blunt-injured organs may be used as models of the normal human spleen.

Keywords Blunt-injured spleen · Gunshot-injured spleen · Human · Morphometry · Stereology · Splenic tissue compartments

Abbreviations
PALS periarteriolar lymphoid sheath

Introduction

The spleen shows a delicate microarchitecture organized in conjunction with its vascular network. This organ plays a unique role among lymphatic organs. The special functional capacity of the spleen can be ascribed to the distinctive organization of its vascular pathways that expose the blood-borne antigens and worn-out erythrocytes to various splenic effector cells [11]. This special structure endows the spleen with the role of a sole blood filter of the body. Other lymphatic organs cannot substitute this function of the spleen and if it is removed from the organism (for example, after the traumatic rupture), such patients show the immunologic and hematologic consequences [10, 14, 16].

The spleen is composed of several tissue compartments, all of which are populated with distinct lymphoid and stromal cells [11]. Various splenic functions are carried out by these compartments and they may be substantially changed, for example during infection [2, 6]. Thus, in addition to cells and molecules expressed by them, the knowledge on structure and volumes of splenic tissue compartments is essential for complete understanding of

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immune or pathological processes in this organ. However, the histoquantitative studies of all tissue compartments of the human spleen are very few. Earlier, we performed a detailed stereological study of the spleen surgically removed after rupture caused by blunt injury and spleen extirpated during surgical treatment of early stage carcinoma [12, 13]. Our work has shown that traumatically ruptured spleen, in comparison to the spleens removed during surgical treatment of early stage carcinoma, shows significantly enlarged perifollicular zone and periarteriolar lymphoid sheath (PALS) [12, 13]. We believed that these differences could either reflect the immune stimulation of the blunt-injured spleen (which alters the structure and consistency of this organ making it more prone to rupture after blunt trauma) or the deterioration of the immune system as a consequence of the malignant disease. To clarify this issue we tried to select a presumptive model of immunologically unstimulated spleen to make comparison with the blunt-injured spleen. We believed that the gunshot-injured spleen could be used for this purpose. In general, animal experiments show that it usually takes several days for immune cells to initiate the defensive reactions and start migration toward the appropriate tissue compartments [7–9]. Comparable kinetic studies of immune reactions and related structural changes in the human spleen are not available in the literature. However, it is known that it takes 180–190 h for the histomorphometric changes to occur in the human spleen during the lethal septicemia [6]. Therefore, we felt that the time period (maximum 12 h, mostly less) between the gunshot injury and surgical removal of the spleen in our study was short enough to prevent histomorphological changes due to the immune reactions induced by the injury itself.

Our study shows that there are no differences between blunt-injured and gunshot-injured spleen. Furthermore, taken together with our earlier results [12, 13], our present data show that the volumes of all tissue compartments of the human spleen remain strikingly constant across various genetic backgrounds.

Materials and Methods

Source of Material

After approval from the institutional review board, the blocks of splenic tissue were obtained from the archives of the University of Maryland Medical Centre, Baltimore, Maryland, USA. All patients were male. They did not have a medical history of illness and, therefore, have been considered healthy. Tissue samples of spleen surgically removed after the blunt injury (n=7), without signs of hemorrhage or immune reactivity (numerous plasmocytes or granulocytes within the red pulp), were selected. The age range of patients was 19 to 54 years (mean = 34.8). Two patients were Caucasian and 5 were African-American. The spleen surgically removed after the gunshot injury (n=5), were included in the study as a separate group. The age of these patients was between 22 to 41 years (mean = 29.2). Three patients were Caucasian and 2 were African-American. The splenic tissue was surgically extirpated not later than 12 h after the gunshot injury of the patients.

Preparation of Tissue

The slices of splenic tissue were fixed in 10% neutral buffered formalin. The tissue was processed and embedded in paraffin. From each block several 6 μm-thick sections were sampled at 3 different levels of tissue 200 μm apart. Thereafter, the sections were either routinely stained with hematoxylin and eosin or used for immunocytochemical detection of B- and T- lymphocytes to verify the identity of splenic tissue compartments. Briefly, the standard three-step immunoperoxidase method (avidin-biotin) was performed, whereby 3,3′-diaminobenzidine tetrahydrochloride (DAB; Dako, Denmark) was used for detection of peroxidase activity. Mouse monoclonal anti-human CD20 (ab9475 at a 1:50 dilution) and rabbit polyclonal anti-human CD3 (ab5690 at a 1:200 dilution) antibodies (Abcam, Cambridge, MA) were used for demonstration of B- and T-cells, respectively. The sections were counterstained with modified Harris hematoxylin. Appropriate negative (normal rabbit or mouse serum) and positive (colon, lung and tonsillar tissue) controls were performed.

Spleen Histomorphometry

Volume Densities of the Splenic Tissue Compartments

The model-based stereology with point-counting method was used. The volume density of a tissue compartment i (V_{vi}) was calculated from the equation: V_{vi}=Pi/Pt, where Pi=number of points falling on the tissue compartment i, and Pt=total number of points counted on the reference tissue [18]. The following tissue compartments were separated: 1) red pulp; 2) perifollicular zone; 3) white pulp—this tissue compartment was divided in two subcompartments: 3a) follicles and 3b) PALS; 4) marginal zone and 5) connective tissue of trabeculas. All determinations were performed at a magnification of ×100 using lattice with 36 points (B 36). Three sections per spleen were utilized for the analysis using the systematic field sampling technique and 20 fields were inspected on each section. The fields were randomly chosen, but did not involve the subcapsular area which consists predominantly of the red pulp.

Stereological Parameters of the Spleen Follicles

The numerical density of the follicles per 1 mm^3 of splenic tissue (N_{Vf}) was calculated from the following equation: $N_{vf} = 1 \Big/ \beta \cdot K \sqrt{N_{Af}^3 \Big/ V_{vf}}$, 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 ×100 and lattice B 36; β=0.87 (shape factor of the follicles); K=1.06 (factor for the size distribution of the follicles) [18]. The mean follicle diameter ($\overline{D}$) was calculated from the equation: $\overline{D} = 2 \cdot \sqrt[3]{3/4\ \pi \cdot V_{vf} / N_{vf}}$ which is based on the assumption that the follicles have a spherical shape [18].

All measurements were done by an investigator fully blind in regard to the experimental group/cause of the splenic trauma.

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

In all examined samples, i.e., in both the blunt- and gunshot-injured spleen, all tissue compartments were easily distinguished (Figs. 1 and 2a, b). B-lymphocytes were predominantly located in lymphoid follicles (Fig. 2a). Some T-lymphocytes were also present therein, especially in secondary follicles at the border of germinal centers (Fig. 2b). However, their predominant location was the PALS (Fig. 2b), which also contained a substantial number of B-lymphocytes (Fig. 2a). In addition to B-lymphocytes, the marginal zone was populated with T-lymphocytes (Fig. 2a, b).

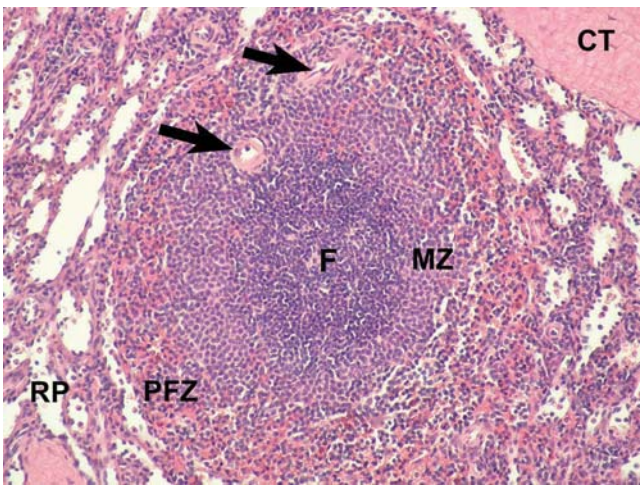

Fig. 1 Section of the human spleen. Male, 25 years old, African-American patient. All tissue compartments are discernible (H&E, ×200). RP=red pulp; PFZ=perifollicular zone; F=follicle; MZ=marginal zone; CT=connective tissue trabeculae; *arrows*=branches of the central artery with periarteriolar lymphoid sheath

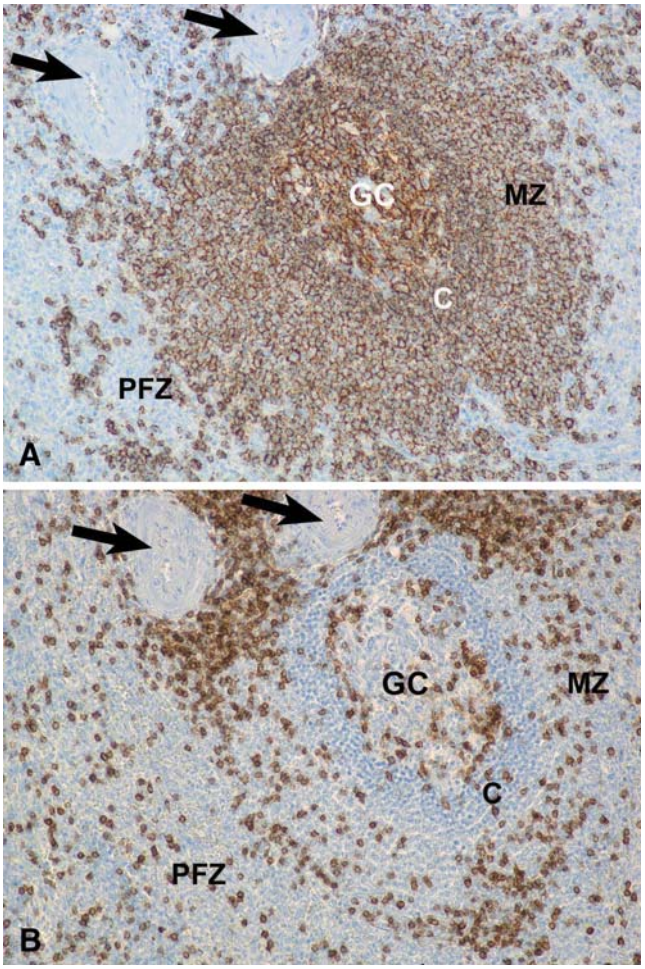

Fig. 2 Adjacent sections of the human spleen. Male, 53 years old, Caucasian patient. **a** B-lymphocytes are predominantly located in the lymphoid follicle, but some are also positioned within periarteriolar lymphoid sheath. Marginal zone is populated with both B- and T-lymphocytes (immunoperoxidase, mouse monoclonal anti-human CD20 antibody, DAB ×200); **b** T-lymphocytes are predominantly located in the periarteriolar lymphoid sheath. They are also present in the secondary follicle, mostly between the germinal centre and follicular corona. Marginal zone is populated with both B- and T-lymphocytes (immunoperoxidase, rabbit polyclonal anti-human CD3 antibody, DAB ×200). PFZ=perifollicular zone; MZ=marginal zone; C=corona; GC=germinal centre; *arrows*=branches of the central artery with periarteriolar lymphoid sheath

In the blunt-injured spleen, the volume density of red pulp and perifollicular zone was 66.0% and 16.6%, respectively. Thus, red pulp and perifollicular zone comprised 82.6% of the splenic volume. Marginal zone represented 6.8% of the splenic tissue and together with the white pulp it comprised 17.6%. The volume density of white pulp was 10.8%, with follicles and PALS comprising 3.5% and 7.3%, respectively. The proportion of connective tissue was 2.5% (Table 1). Various stereological parameters

Table 1 Volume densities (%) of tissue compartments of the spleen (means±SD)

Parameter	V_{vrp}	V_{vpfz}	V_{vmz}	V_{vwp}	V_{vfoll}	V_{vPALS}	V_{vct}
Blunt injury	66.0±7.7	16.6±8.9	6.8±3.4	10.8±2.0	3.5±1.1	7.3±2.0	2.5±1.4
Gunshot injury	64.3±5.7	14.4±5.1	6.9±2.2	11.6±3.6	3.9±1.6	7.7±3.8	2.6±2.5

V_{vrp} volume density of red pulp, V_{vpfz} volume density of perifollicular zone, V_{vmz} volume density of marginal zone, V_{vwp} volume density of white pulp, V_{vfoll} volume density of follicles, V_{vPALS} volume density of periarteriolar lymphoid sheath, V_{vct} volume density of connective tissue

of the follicles of blunt-injured spleen are shown in Table 2. The number of follicles per mm^2 and the numerical density of the follicles were 1.0 and 6.6, respectively. The mean follicular diameter was 223.8 μm. Volume density of the follicles was 0.03.

In gunshot-injured spleen, the volume density of red pulp was 64.3%. The volume density of perifollicular zone was 14.4%. Jointly, they composed 78.7% of the splenic volume, which was not different from the blunt-injured spleen. Marginal zone constituted 6.9% of the tissue. Together with the white pulp it composed 18.5%, which was not different from the values for the blunt-injured spleen. The volume density of white pulp was 11.6%, which was comparable to the trauma group. Follicles were 3.9%, whereas PALS comprised 7.7%, which was similar to the values obtained for the spleen extirpated after traumatic injury. The amount of connective tissue (2.6%) varied greatly from spleen to spleen (Table 1). Various stereological parameters of follicles of gunshot-injured spleen are shown in Table 2. The number of follicles per mm^2 of spleen section was 1.2 and the numerical density of the follicles was 8.1. The mean follicular diameter was 214.0 μm, whereas the volume density of the follicles was 0.04. These values were not different from those obtained in the group of spleen removed after blunt injury.

Discussion

We did not reveal any morphometric difference between the blunt-injured and gunshot-injured spleen—in respect to the volume densities of all tissue compartments and stereological parameters of lymphoid follicles the blunt-injured spleen is comparable to the gunshot-injured spleen. In our study the gunshot-injured spleen was taken as a presumable model of immunologically unstimulated organ, based on numerous kinetic studies which show that it takes much longer than 12 h (the maximum time period between the gunshot injury and removal of the spleen in our study) to initiate the immune activity and morphological changes in the lymphatic organs [7–9]. Thus, our results suggest that both gunshot-injured and blunt-injured organs may be used as models of the normal human spleen.

The blunt-injured spleen did not show the increased volume density of any tissue compartment related to immune reactivity. Still, the increased weight of the blunt-injured spleen has been observed in comparison with the gunshot-injured spleen [3]. This increase may be caused by the profuse interstitial hemorrhage caused by the blunt trauma prior to splenic rupture, which leads to formation of large intraparenchymal hematomas [3, 4] with resulting weight increase [3]. Such tissue regions were microscopically observed, but were carefully avoided in our study and were not used for histoquantitative analyses. If this was not possible due to the large extent of hemorrhagic regions, such splenic samples were excluded from the study.

Earlier, we have shown that, in comparison to the blunt-injured spleen, the spleen extirpated during treatment of cancer patients has significantly reduced perifollicular zone and PALS [13]. It is very likely that these changes reflect the functional and structural deterioration of the immune system as a consequence of malignant disease [12]. This view is confirmed by studies which show the impaired function of thymic stromal cells and arrested thymocyte development in tumor-bearing mice [1, 17]. Also, the frequencies and activities of splenic T-helper-1 lymphocytes are reduced during tumor growth [5, 15]. Thus, the complex functional alterations of the immune system in

Table 2 Stereological parameters of splenic lymphoid follicles (means±SD)

Parameter	N_{Af} (mm^{-2})	N_{Vf} (mm^{-3})	$\overline{D}$(μm)	V_{vfoll} (mm^3/mm^3)
Blunt injury	1.0±0.4	6.6±3.6	223.8±43.1	0.03±0.01
Gunshot injury	1.2±0.3	8.1±2.7	214.0±46.0	0.04±0.02

N_{Af} number of follicles per mm^2 of section area, N_{Vf} numerical density of follicles per mm^3, $\overline{D}$ mean follicular diameter, V_{vfoll} volume density of follicles

tumor-bearing organism are likely to sum up structurally as a reduction of PALS in cancer patients described in our earlier studies [12, 13].

The data obtained in this study are identical to our earlier results regarding the volumes of splenic tissue compartments [12, 13]. This is very significant, because the patients included in these studies are racially disparate: Semitic/Maltese [12, 13], Caucasian and African-American [this study]. The mechanisms controlling the development, organization and maintenance of splenic tissue compartments have been extensively studied in recent years [11]. Our present results show that in the human spleen the volumes of tissue compartments and their subcompartments are very stringently controlled and kept stable under normal conditions. This is further underscored by the fact that the volume density of lymphoid follicles is maintained constant despite the variations in their diameter registered earlier [12, 13] and in this study. The decreased size is compensated by the increase in number of follicles [this study], which keeps their volume density constant and identical to that observed earlier [12, 13].

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