

**PH.D.THESIS**

# **Changes in the immune system of splenectomised and spleen autotransplanted mice**

**Sándor J. Sipka Jr.**



**Medical and Health Science Center  
University of Debrecen  
Faculty of Medicine  
Department of Operative Techniques and Surgical Research**

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## **1. INTRODUCTION**

Splenectomy was already known in the ancient Greek times, when it was performed in cases of the extremely enlarged spleen caused by malaria. The first authentic splenectomy was made by Zaccarello in 1549. Later splenectomy spread widely as it was thought that the spleen would be a functionless organ. This viewpoint changed as King and Schumacher described the overwhelming postsplenectomy infection syndrome (OPSI-syndrome) on five previously splenectomised children.

Nowadays the saving of spleen, or at least a part of it, became daily routine in the operative medicine. In the cases of smaller injuries -or injuries parallel with the anatomic borders of the segments- conservative management can be successfully performed. In such cases mattress or figure-of-8 sutures can be used as well as bioplasts or tissue adhesives. Partial splenectomy is also an opportunity for saving at least a part of it.

In case of the unsaveable spleen splenectomy has to be performed. In such situations the need for replanting / transplanting of splenic tissue arises. Since decades the greater omentum has been recognized already as a potential place for transplantation, as after traumatic injuries spontaneous replantation of the splenic tissue can be observed in it, called the splenosis. The technical aspects of heterotopical efficient splenic autotransplantation with various amounts of healthy spleen tissue have been widely reported from several research groups, however just a few of them were used in human practice.

Using the greater omentum, a “spleen apron method” was earlier developed by Furka and his coworkers on dogs in our institute in 1989. According to this method, several slices of the spleen (about 2-3 mm thin and with the diameter of 2-3 cm, representing about 10-15 % of the original splenic mass) were inserted into the two layers of greater omentum (forming an “apron”). By this technique, the splenic chips remained in their places. They were nourished through a large surface, and the prevention of formation of adhesions was observed. These technique has been introduced to the clinical practice first in Debrecen, and up to now more than three hundred patients were treated in this way in Hungary.

This method was adapted also for mice by a microsurgical technique by Mikó and her coworkers in 2001. Applying this in the current work we had the opportunity to follow up the morphological and functional changes after spleen autotransplantation in inbred mice.

## **2. Objectives**

We suppose to find and demonstrate significant immunological and hematological changes of spleen autotransplanted, sham operated and control inbred mice 2 and 8 months after the operations.

We aimed to perform the following laboratory investigations, measuring:

- 1) The changes in the numbers and the ratios of the corpuscles of the peripheral blood;
- 2) The changes in the numbers and the ratios of the lymphocyte subsets;
- 3) The changes in the serum levels of immunoglobulins and complement factors;
- 4) The changes in the activity of the peripheral phagocytes;
- 5) By light microscopic analysis the splenic autotransplants using haematoxylin-eosin staining and immunohistological tools.

### **3. MATERIALS AND METHODS**

#### **3.1. Animals**

Two months old male and female Balb/c mice (body weight:23–26 g, n=126) were used for the study. The animals were operated two and eight months before the measurements, and the laboratory measurements were carried out simultaneously. This means that the animals were four and ten months old at the time of the measurements. The hematological and immunological examinations were carried out on 96 mice, and 30 additionally were dedicated for histology.

#### **3.2. Surgical operations**

The animals were anesthetized with an intraperitoneal injection of pentobarbital (35 mg/kg) and were operated using an operating microscope in clean but not sterile conditions. The animals were divided into four groups based on the interventions:

1. Control: no surgical intervention was made;
2. Sham operation: lifting out and reposition of spleen after a midline incision;
3. Splenectomy splenectomy was performed after a midline incision;
4. Spleen autotransplantation: after removing the spleen, we cut 5 small pieces of the splenic tissue -called “chips” – with the size of 2×2×2 mm (about 10-15 % of the original size) and we placed them into a Ringer-lactate saline solution at room temperature. The greater omentum was raised and after forming 5 omental nests in the

first layer of the greater omentum, we placed the chips inside. The nests were signed and fixed with 7/0 Prolene stitches.

The abdominal wall was closed in two layers (peritoneal–aponeurotic plane and skin) in all operated cases.

Experimental groups:

I. Two months after the operations:

Group 1 - Control (C-2)

Group 2 - Sham operation (Sh-2)

Group 3 - splenectomy (SE-2)

Group 4 - autotransplantation (AU-2)

II. Eight months after the operations:

Group 5 - Control (C-8)

Group 6 - Sham operation (Sh-8)

Group 7 - splenectomy (SE-8)

Group 8 - autotransplantation (AU-8)

### **3.3. Macroscopic examinations**

We examined the autotransplants and the vessels running towards them by an operating microscope. The samples were taken only from those animals in which all five splenic transplants were found.

### **3.4. Laboratory measurements**

Blood was taken under pentobarbital anesthesia (35 mg/kg) by an intracardial puncture after thoracotomy.

The quantitative and qualitative cell count determinations were carried out by a microcell counter (DiaTerm Abacus, Hungary) from heparinized blood (7 U/ml).

The subsets of lymphocytes were measured by a flow cytometer (FACSCalibur, Becton-Dickinson, USA) using the following antibodies: PE anti-mouse CD3; FITC anti-mouse CD 19; PE anti-mouse NK1.1 (CD-56) (Pharmingen, USA).

Immunoglobulin and complement components were determined by a laser nephelometer (Behring, Germany), using the following reagents: IgM, IgA, C3, C4 (DAKO, Denmark), and IgG (Behring, Germany) from sera stored at -20 °C.

The activity of peripheral blood phagocytes (mainly of neutrophil granulocytes) was characterized by chemiluminescence (RFU= Relative Fluorescence Unit) can be determined in heparinised whole blood stimulated by zymosan(baker yeast cell wall derivate - Sigma, USA) amplified by luminol (Sigma, USA) and detected by a luminometer (Berthold, Germany).

### **3.5. Microscopic examinations**

*Haematoxylin-eosin staining.*

*Immunohistology:* The immunohistological examinations were performed using rat antibodies specific for mouse endothelium and stroma cells. They were carried out Department of Immunology and Biotechnology at the University of Pécs.

### **3.4. Statistic analysis**

The results are expressed as means and standard deviations (mean $\pm$ S.D.). When the distribution of data was normal, the one-way ANOVA test was used for the inter-group comparisons. If the result of ANOVA test was significant, the Dunett test was applied to compare the means in the pairs. When the distribution was not normal, the Kruskal–Wallis test was applied. P values smaller than 0.05 were considered statistically significant.

## **4. RESULTS**

### **4.1. Macroscopic examinations**

Before sample taking, we examined the autotransplants with opened eyes and using operating microscope. Normally we found enlarged vessels running towards them, showing their survival. In cases-when the autotransplants didn't survive- we could just recognize the suture material used for closing the nest. We only used the data of animals with five surviving chips.

Eight months after the operations the splenic transplants still survived, moreover they enlarged their size about twice than that of their original sizes.

### **4.2. Results of the laboratory measurements**

#### **4.2.1.1. Changes in the mean corpuscular volume**

Two months after the operations, we found that the main erythrocyte volume -MCV- was significantly higher in the splenectomised (SE-2) group than in the controls (C-2).

If we compared the two groups of control mice, the MCV of the erythrocytes were significantly increased in the older (C-8) than in the younger (C-2) animals. There was no difference among the MCV values of the animals eight months after the operations.

#### **4.2.1.2. Changes in the number of lymphocytes**

Two months after the operations there were no difference among the groups. On the other hand, eight months after the operations, we found a significant decrease in the splenectomised group compared to the controls.

### **4.2.2. Changes in the lymphocyte subsets**

#### **4.2.2.1. Changes in the number of CD3+ T lymphocytes**

Two months after the operations there was no difference in the number of circulating CD3+ T -cell in the peripheral blood.

On the other hand, eight months after the operations the number of T -cells was significantly decreased in both groups of operated (splenectomised SE-8 and autotransplanted AU-8) animals compared to the controls (C-8). However, the autotransplanted animals had about 15-20 % higher number of CD3+ T -cells than the splenectomised animals.

#### **4.2.2.2. Changes in the number of CD19+ B**

Two months after the operations the number of circulating CD19+ B -cells increased significantly in the splenectomised (SE-2) and decreased in the autotransplanted (AU-2) animals compared to the controls (C-2).

Eight months after the operations, however, in both groups of operated animals (SE-8 and AU-8) the number of B cells was diminished compared to the controls (C-8) but only the decrease measured in the splenectomised mice was significant. The value of AU-8 was about 15-20 % higher than in the splenectomised animals (SE-8).

### **4.2.3. Serological changes:**

#### **4.2.3.1. Changes in the serum levels of immunoglobulin IgM**

The level of serum IgM was significantly decreased two months after the operations both in the splenectomised (SE-2) and in the spleen autotransplanted (AU-2) groups compared to the controls (C-2). The tendency of falling went on in the splenectomised animals (SE-8), but it remained at the two months level in the autotransplanted animals (AU-8).

We didn't find significant differences in the serum levels of IgG, IgA, C3 and C4 in any examined groups.

#### **4.2.4. Changes in the phagocytes**

##### **4.2.4.1. Changes in the number of the granulocytes**

Two months after the operations we found an increase in the granulocyte number of the splenectomised group (SE-2) compared to the controls.

The number of the granulocytes significantly elevated in the older group of control mice (C-8) compared to the younger controls (C-2).

Eight months after the operations, in splenectomised (SE-8) group the number of the granulocytes was significantly elevated compared to the controls (C-8). The elevation was smaller in the autotransplanted group (AU-8).

##### **4.2.4.2. Changes in the phagocyte activity**

Surprisingly in activity of phagocytes we found the highest level of activation not in the splenectomised group (SE-8), where the number of the neutrophil granulocytes was the highest, but in the autotransplanted group (AU-8).

#### **4.3. Microscopic examinations**

##### **4.3.1. Haematoxylin-eosin staining**

Both two and in the eight months after the autotransplantations the splenic chips showed normal splenic structure with trabecules, red and white pulp, showing the splenic chips could survive and stay active there.

##### **4.3.2. Immunohistology:**

The blood flow of the splenic chips changed. The original splenic artery ceased, and new vessels started to grow in from the greater omentum.

Using the IBL-7/22-es antibody what is an endothelium and reticular fibroblast marker, we found out, that the central artery disappeared for the eight months.

Using the MAdCAM-1 antibody, the marker of marginal sinus of the mice showed altered staining pattern compared to the sham operated, ones demonstrating changes in the B - cell homing.

There were changes in the T and B cell zones, namely, the B -cell expanded at about 30 % of its original size, and the area of T cells zone decreased about 50%. The follicular dendritic cells appeared in the B- cell zones, as usual.

We followed up the pattern of two different subsets of the macrophages, but they didn't show changes compared to the sham operated control.

## **5. DISCUSSION**

In the current work we present some significant changes in the peripheral distribution of CD3+ T and CD19+ B cells, in the level of serum IgM and in the activity of peripheral phagocytes of autotransplanted mice compared to the splenectomised animals suggesting that a surgical attempt for the partial restoration of spleen after splenectomy may have some immunological benefits.

The immune competence of the spleen is crucially dependent on the tissue architecture and the recirculation efficiency of lymphoid cells to and from the spleen. In our histological analyses performed eight months after the autotransplantation the lymphoid tissue structure was clearly compartmentalized into red pulp and lymphocyte-rich white pulp, latter with a slight Bcell dominance compared to controls

The main point of this work is that some functional and immunological activity of the grafted spleen slices can be observed in the inbred mice.

The first conclusion drawn from the data of significant alterations is that there are time dependent differences in the changes of immune system of mice, especially in the peripheral distribution (in the number) of circulating B cells and neutrophils. In addition, the alterations caused by splenectomy or autotransplantation also can be different two or eight months after the operations.

It was surprising that the absolute number of CD3+ T cells was almost the same in the four groups two months after the operations. On the other hand, a striking fall of these cells was observed in the two operated groups eight months after the interventions. In addition, it



was also not negligible that the number of T cells was slightly (although not significantly) higher in the autotransplanted than in the splenectomised animals.

In the case of B cells the directions of changes were different in the animals of different ages. Whereas the number of B lymphocytes was significantly elevated in the splenectomised mice compared to the controls two months after the operations, there was a rather great fall in the number of these cells in the autotransplanted mice. The increased bone marrow activity of splenectomised mice could be even better reflected by the remarkable neutrophil granulocytosis observed simultaneously. We think that the reason(s) of the rather great differences in the number of circulating B and T lymphocytes in the autotransplanted animals can be related to the special and time dependent change in the “homing” circumstances for them in the “chips” as it was demonstrated in our in vivo cell transfer experiments mentioned above. It has to be stressed, however, that though eight months after the interventions, the number of circulating B cells was decreased in both splenectomised groups of animals, in the autotransplanted mice, this fall was less than in the splenectomised animals, showing a slightly better capacity for the production of immunoglobulins.

The production of IgM is crucial after splenectomy from the point of view of “overwhelming postsplenectomy infection” (OPSI) in the surgical practice. Therefore, it could be regarded to be the most important finding in this work that in the autotransplanted animals we measured a significantly higher level of serum IgM than in the splenectomised mice eight months after the operations. This change could be in parallel with the slightly elevated number of B cells in these mice helping possibly a better antimicrobial defense than that of splenectomised animals, where the impaired production of IgM was reflected both two and eight months after the interventions in spite of the significantly high number of peripheral B cells in the group of two months old grafting. The improved phagocytic activity in the autotransplanted mice compared to the splenectomised one's is also an advantage of spleen replantation.

It was common in the present experiments that in the autotransplanted animals the values of T and B cell number, the serum IgM level and the phagocytic activity were higher than in the splenectomised mice eight months after the operations reflecting the functional activity of replanted spleen tissue, however, it represented only the 10–15% of original mass.

This approach allows us to declare that possibly a direct and quantitative linkage can exist between the successfully implanted mass of spleen and its positive influence on the number of T and B cells, circulating IgM and peripheral phagocytic activity compared to

splenectomy. Supposedly, the larger the mass of successfully transplanted spleen, the better is the clinical effect.

## **6. NEW RESULTS**

In the current work we monitored the hematological and immunological changes after the spleen-autotransplantation in inbred mice, and we found the following changes:

- 1.) The autotransplanted spleen chips have some positive effect on the filtration of the erythrocytes, the ratio and number of the T- and B- lymphocytes, the phagocytic activity of neutrophil granulocytes, and the level of IgM playing a crucial role in the antibacterial defense.
- 2.) The blood circulation of spleen chips replanted into the greater omentum alters. The central artery disappears, the arrangement of the follicles, the structure of red and white pulps change. a.) the decrease in the number of T- lymphocytes both in the spleen and in the periphery, b.) decreases in the number of B-lymphocytes in the periphery but increases in the follicles.

### 7.1. Articles of the thesis

1. **Sipka S. jr.**, Bráth E., F. Tóth F., Fábián Á., Krizsán Cs., Baráth S., Sipka S., Németh N., Bálint A., Furka I., Mikó I. (2006) Distribution of peripheral blood cells in mice after splenectomy or autotransplantation.  
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### 7.2. Articles not related to the thesis

4. Mikó I., Bráth E., Németh N., F. Tóth F., **Sipka S. jr.**, Kovács J., Sipka S., Fachel J., Furka A., Furka I., Zhong R. (2003) Hematological, hemorrheological, immunological and morphological studies of spleen autotransplantation in mice: preliminary results.  
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