Functional Hypersplenism in Mice Induced by Adoptive Transfer of Syngeneic Spleen Cells

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Investigation of peripheral blood cell count alterations in cases with hypersplenism, and an understanding of the relationship between splenic function and hematopoietic cell production require suitable experimental animal models. Previously described methods are either traumatic or require surgical intervention. We suggest a relatively simple method for achievement of a state mimicking hypersplenism in mice by intraperitoneal inoculation of syngeneic spleen cells. Mice were inoculated intraperitoneally with 3×10^7 splenocytes suspended in 0.3 ml phosphate buffered saline (PBS). After 2 months, the inoculated animals showed a progressive decrease in the peripheral white blood cell (WBC) counts and hyperplastic bone marrow that persisted until the experimental end point (7 months). Five days after inoculation of splenocytes stained with carboxy-fluorescein diacetate succinimidyl ester (CFSE), the majority of the stained cells was present in the peritoneal cavity (33%) and in the liver (13%), whereas the percentage of stained cells in the peripheral blood and the spleen cell suspension was negligible. The mitogen response of the peripheral blood mononuclear cells (PBMC) from treated mice to concanavalin A (Con A) remained unaltered. Splenocyte-inoculated mice that were further splenectomized did not show leukocytosis after splenectomy, as was observed in animals in which the spleen was removed without any pretreatment. The lack of any signs of discomfort in animals from the study group, in comparison with the visibly ill appearance and even death of mice in which hypersplenism was achieved by repeated injections of methylcellulose (MC), which served as controls, favors the convenience of the method. Exp Biol Med 231:112–116, 2006

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Introduction

The spleen is a mysterious organ with functions that still intrigue both clinicians and researchers. Removal of the spleen results in changes in the immune responses of the organism, including marked elevation of the peripheral blood cells, an effect applied as a therapeutic method in certain blood disorders. On the other hand, patients with hypersplenism frequently associated with splenomegaly show a decrease in peripheral blood cell counts and in hyperplastic bone marrow. The relationships between splenic size and function and variations in peripheral blood cell counts, as well as the production of hemopoietic cells in the bone marrow, have yet to be elucidated, and require suitable animal models. Attempts to induce experimental hypersplenism in animals were made a few decades ago; however, they have been overlooked in recent years. The methods to induce experimental hypersplenism are intricate. Hypersplenism in mice and rats has been achieved by repeated intraperitoneal injections of methylcellulose (MC), resulting in spleen enlargement, anemia, leucopenia, and thrombocytopenia (1–4). Splenic-vein ligation in rats has been reported to induce secondary hypersplenism, expressed by lower red blood cell and platelet counts (5). However, these methods encompass certain drawbacks—injection of MC causes not only splenomegaly, but also enlargement and histologic changes in other organs, such as the liver, kidneys, adrenal glands, and lungs (4). For splenic-vein ligation, the animals have to be surgically treated, a procedure linked with stress and blood loss.

Therefore, the purpose of the present study was to induce experimental hypersplenism in mice in a relatively nontraumatic way *via* intraperitoneal inoculation of syngeneic spleen-derived cells. Changes in peripheral white blood cell (WBC) counts and bone marrow cellularity characteristic for hypersplenism served as an indication for the usefulness of the method. In addition, the proliferative response of the peripheral blood mononuclear cells (PBMC)

to concanavalin A administration was evaluated in these animals.

Materials and Methods

Animals. The Animal Investigation Committee of the Medical Center approved the study. The experiments were performed in accordance with the guidelines for the care and use of laboratory animals forwarded by the local Committee on Animal Care. The animals were kept in a ventilated and temperature-controlled environment, they were placed on a standard diet, and received food and water *ad libitum*. Fifty 2-month-old Balb/c age-matched female mice were included in the study. The animals were divided into three groups: 20 mice were inoculated with splenocytes, 10 were injected with MC, and 20 mice served as controls. At the end of the study, the mice were killed by decapitation and the spleens of animals from the first two groups were removed and weighed, and the peritoneal cavity was inspected. For evaluation of splenocyte migration, the liver and the mesenteric lymph nodes were minced through a metal mesh to achieve a single-cell suspension.

Splenocyte Assay. Spleens removed from untreated mice not included in the experimental groups were minced through a metal mesh to obtain single-cell suspensions. The cells were washed twice in phosphate-buffered saline (PBS) and counted. For each mouse, 3×10^7 splenocytes suspended in 0.3 ml PBS were injected intraperitoneally. Five months later, 10 animals underwent total splenectomy, performed with the animal under anesthesia with ether. The abdominal wall was opened, the spleen blood vessels were ligated, and, after removal of the spleen, the abdomen was sutured shut.

MC Assay. For the MC assay, 2.5 g of MC (400 aquiipois; Fisher Scientific Co. Fairlawn, NJ) were dissolved in 100 ml of distilled water at 100°C and stored at 4°C. Mice were intraperitoneally injected with 0.3 ml of 2.5% MC solution twice a week for 8 weeks, and, thereafter, once a week for an additional 6 weeks.

Blood Counts. Blood samples were collected from the tail vein and cell counts were performed using a Technicon H-2 cell counter (Bayer, Leverkusen, Germany).

Bone Marrow. Bone marrow was obtained from the hind femur and tibia by introducing a 26-gauge needle into the lumens and expulsing the material with a syringe containing PBS. Bone marrow smears were stained using May Grünwald-Giemsa staining and examined with a light microscope under oil immersion by an experienced hematologist who had no knowledge regarding the experiments' details.

Mitogen Response. Peripheral blood mononuclear cells were isolated using Histopaque gradient centrifugation (Sigma Chemical Co., St. Louis, MO). The cells were suspended in RPMI-1640 medium containing 1% penicillin, streptomycin, and nystatin, and supplemented with 10% fetal calf serum. Peripheral blood mononuclear cells were divided into 0.1-ml aliquots, and each aliquot was placed into one well of a flat-bottom 96-well plate (Nunc, Rochester, NY) containing 0.1 ml of 10 mg/ml concanavalin A (Con A; Sigma). The cultures set up in triplicates were incubated for 3 days. At 18 hrs before harvesting, 1 μCi/well of 5 Ci/mmol methyl-^{3}H-thymidine (Amersham, Little Chalfont, Buckinghamshire, England) was added. Radioactivity was measured with a LKB liquid scintillation counter model 3380.

Detection of Cell Migration. For examination of cell migration, staining of splenocytes with carboxyfluorescein diacetate succinimidyl ester (CFSE) was performed using the fluorescein-based dye (Molecular Probes, Inc., Eugene, OR), according to the method of Weston *et al.* (6). Briefly, splenocytes obtained as described in "Splenocyte Assay" were washed in PBS, sedimented by centrifugation, and suspended in 1.5 ml of 5-μ*M* CFSE solution. The cell suspension was incubated for 60 mins at 37°C. At the end of the incubation period, the cells were washed twice and suspended in PBS. Each mouse was injected intraperitoneally with 30×10^6 CFSE-labeled splenocytes suspended in 0.3 ml PBS. After 5 days, labeled cells were counted in bone marrow, peripheral blood, liver, spleen, and mesenterial lymph nodes cell suspension, as well as in peritoneal fluid obtained by washing of the peritoneal cavity. Stained splenocytes were counted with a fluorescence-activated cell sorter (Calibur flow cytometer, Immunofluorometry Systems; Becton-Dickinson, Mountain View, CA), by passing them at a rate of 1000 cells/sec, using physiologic saline as the sheath fluid. A 488-nm argon laser beam served as the light source for excitation. Emission from 10,000 cells was measured, applying logarithmic amplification, and calculated using CellQuest software (CellQuest, St. Louis, MO). Background noise was determined using unlabeled cells.

Statistics. Statistical analysis was performed with paired and independent Student's t tests. Values are expressed as mean ± SE.

Results

Animals injected with splenocytes did not show any signs of discomfort. Their mean body weight did not differ from that of the controls. On the other hand, animals injected with MC seemed weak, lost weight, and showed visible signs of illness, a reason for reducing the MC dose. By the third month of MC administration, two mice died.

Spleen Size and Peritoneal Cavity. Four months after splenocyte administration, the mean size of the spleen did not differ significantly from that of the controls (0.165 ± 0.153 g vs. 0.146 ± 0.13 g, respectively). On the other hand, 3 months after MC administration, the spleens were found to be extremely large, with a mean weight of 1.265 ± 0.1 g ($P < 0.001$). The peritoneal cavity and organs from splenocyte-inoculated animals seemed normal, whereas, in MC-treated animals, the liver seemed slightly enlarged and a few hemorrhagic spots were noted in the peritoneum.

Table 1. Summary of Pertinent Hematologic Data

	WBC (× 10^6 cells/ml)	Lymphocytes (%)	Monocytes (%)	Polymorphonuclear cells (%)	Hemoglobin (g%)	Platelets (× 10^9 cells/ml)
Controls	19.8 ± 0.4	90.1 ± 0.5	7.7 ± 1.0	1.8 ± 0.2	16.7 ± 0.2	1.14 ± 0.5
After splenocyte administration						
1 month	21.2 ± 1.3	92.2 ± 0.8	6.9 ± 0.4	1.5 ± 0.2	15.8 ± 0.3	1.12 ± 0.03
2 months	13.6 ± 0.8**[a]	90.3 ± 1.3	8.5 ± 1.2	1.2 ± 0.1	16.4 ± 0.2	1.13 ± 0.04
3 months	12.4 ± 0.5**	88.0 ± 0.9	10.1 ± 1.0	1.9 ± 0.2	14.3 ± 0.3	0.94 ± 0.02
4 months	11.1 ± 0.5**	84.5 ± 1.7	12.6 ± 1.3	3.0 ± 0.4	16.6 ± 0.2	1.15 ± 0.03
7 months	11.3 ± 0.6**	84.0 ± 1.2	11.2 ± 1.3	4.8 ± 1.0	16.7 ± 0.7	1.09 ± 0.02
MC-injected mice						
1 month	21.0 ± 1.4	83.1 ± 1.0	13.6 ± 0.9	3.3 ± 0.5	12.9 ± 0.7	0.71 ± 0.07*
2 months	28.6 ± 1.8**	62.3 ± 3.3**	20.8 ± 1.6**	11.9 ± 1.7**	11.8 ± 0.3**	1.05 ± 0.07
3 months	48.7 ± 12.3**	37.1 ± 8.1**	27.4 ± 2.7**	35.2 ± 7.3**	9.9 ± 0.5**	0.92 ± 0.17
Splenectomy						
2 months	38.5 ± 1.5**	94.2 ± 0.5	4.8 ± 0.5	1.1 ± 0.03	16.1 ± 0.2	1.48 ± 0.03**
Splenocyte administration and splenectomy						
5 + 2 months	19.8 ± 0.4[b]	75.3 ± 2.5	14.9 ± 1.0	9.8 ± 1.9	15.8 ± 0.4	1.2 ± 0.05

[a] Significantly different from the control group (* $P < 0.05$; ** $P < 0.001$).
[b] Significantly different from mice after splenocyte administration ($P < 0.05$) or 2 months after splenectomy ($P < 0.001$).

WBC Counts (Table 1). One month after splenocyte administration, the total WBC count in the peripheral blood was similar to that of the controls. However, beginning at Month 2, and up to 4 months after splenocyte inoculation, the WBC count gradually decreased to 13.6 ± 0.8, 12.4 ± 1.0, and 11.1 ± 0.5 × 10^6 cells/ml, respectively ($P < 0.001$) and remained at that level up to 7 months after beginning the procedure. No differences in hemoglobin level, platelet count, or differential count were observed between the study and control group after splenocyte administration.

After MC administration, the hemoglobin level decreased and a gradual increase in the total WBC count was observed. The percentage of lymphocytes showed a significant decrease, whereas the percentages of the monocytes and polymorphonuclear cells were increased.

Splenectomy caused an increase in WBC count ($P < 0.001$). However, splenocyte-inoculated mice that underwent splenectomy 5 months later had a lower WBC count 2 months after the operation ($P < 0.001$). Although this number was higher than that in animals injected with splenocytes only, it did not differ significantly from the WBC count in the control group.

Bone Marrow. Bone marrow of splenocyte- and MC-administered mice showed marked cellular hyperplasia, mainly of the white blood series, with a shift to the left caused by a marked increase in the number of immature cells. The red blood and megakaryocytic series were also hyperplastic.

Mitogen-Induced Proliferation (Fig. 1). Five months after splenocyte administration, the proliferative response of PBMC to Con A administration from inoculated animals was similar to that of controls (15,300 cpm ± 900 vs. 18,540 cpm ± 2,690, respectively).

Two months after splenectomy, the proliferative response of PBMC to Con A was higher than that of the control group (29,932 cpm ± 1,520; $P < 0.001$). The mitogen response of PBMC from mice inoculated with splenocytes and splenectomized 5 months later was lower (11,825 cpm ± 1,470) than that of cells from splenectomized animals ($P < 0.001$) and controls ($P < 0.003$), but it did not differ from the cell response in mice injected with splenocytes only.

Cell Migration. Figure 2 shows the percentage of CFSE-stained cells in various tissues 5 days after their administration. The majority of labeled splenocytes (33%) were found in the peritoneal cavity, followed by the liver (13%), whereas their percentages in the peripheral blood and spleen-cell suspension were negligible. No labeled cells were found in the bone barrow or lymph node cell suspension.

Discussion

The classic definition of hypersplenism consists of splenomegaly, pancytopenia, and bone marrow hyperplasia, and improvement of these criteria after removal of the spleen (7). However, the decrease in number of the peripheral blood cells may encompass one or more of these elements (7, 8). The results of the present study show that intraperitoneal inoculation with syngeneic splenocytes in mice results in development of peripheral blood leukopenia, a feature characteristic for hypersplenism. The marked hyperplasia of bone marrow cellularity, with increased number of immature elements, is compatible with the assumption that splenocyte-inoculated mice became hypersplenic. Because the majority of mouse peripheral WBCs are lymphocytes, it was difficult to assess a change in differential count after splenocyte inoculation. A hypothesis to explain the mechanism by which the inoculated

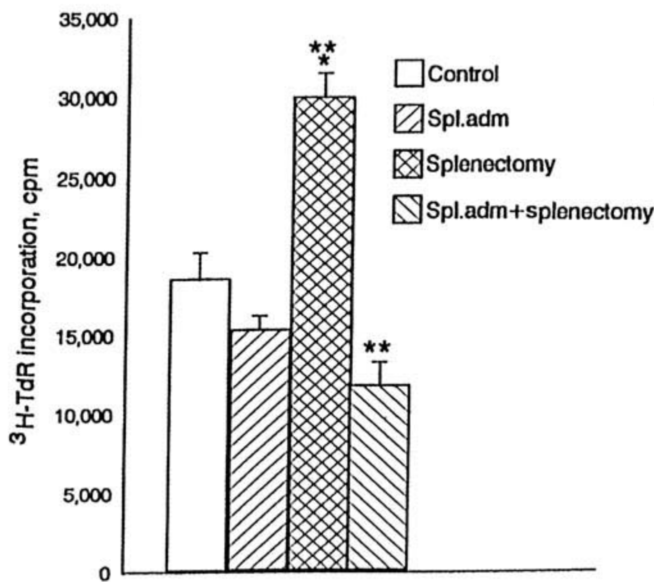

Figure 1. Response of PBMC to Con A administration. Although the proliferative response of cells from splenocyte-inoculated animals (Spl. Adm.) did not show any significant change, cells from splenectomized mice showed a marked increase in that function. On the other hand, cells from splenectomized mice that further underwent removal of the spleen showed a significantly decreased proliferative response to Con A administration. ** $P < 0.003$; *,* $P < 0.001$.

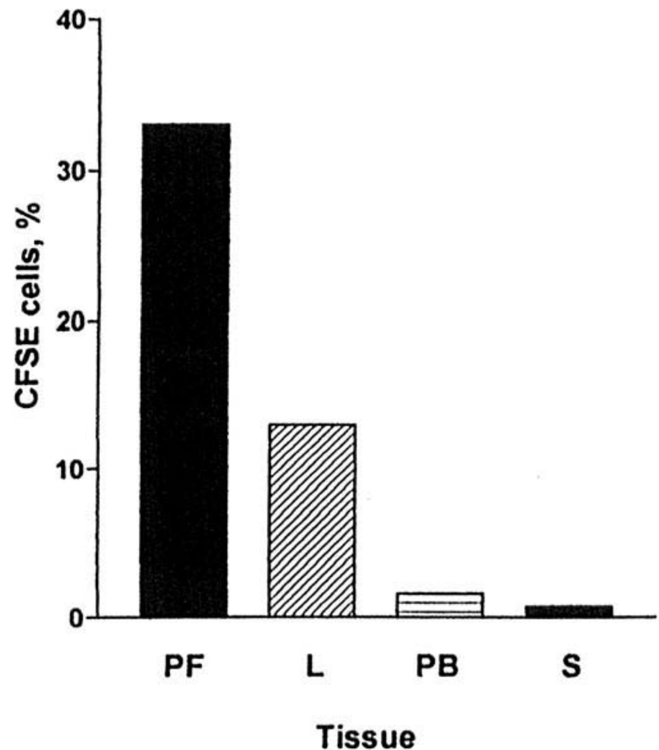

Figure 2. Percentage of CFSE-stained splenocytes in various tissues 5 days after their inoculation into the peritoneal cavity. PF, peritoneal fluid; L, liver; PB, peripheral blood; S, spleen.

splenocytes induced a state of hypersplenism without spleen enlargement is that the inoculated cells seeded the peritoneum and acted as microsplenunculi. The finding of a high percentage of labeled cells in the peritoneal fluid is consistent with this assumption. Failure to observe formation of small foci of splenic tissue in the peritoneal cavity with the naked eye does not exclude the possibility of a diffuse dissemination of the inoculated cells in the peritoneum, similar to the process observed in splenosis. The observed lack of leukocytosis after splenectomy in our study, in mice previously inoculated with splenocytes, supports this supposition. Similarly, Palmer *et al.* (8) found that transplantation of approximately 10% of the removed spleen into the abdominal wall of rats was sufficient to prevent leukocytosis after splenectomy. The higher labeled-cell percentage in the liver, next to the peritoneal cavity, may be explained by better drainage of abdominal organ circulation *via* the portal vein.

Spleen autotransplantation has been performed in both humans and rats by suture of small pieces of the excised organ into an omental pouch (9, 10). However, this method requires surgery, a traumatic procedure avoided in our study. The question of how an enlarged spleen affects the production of blood cells in the bone marrow remains unresolved. However, the hyperplastic bone marrow observed in cases with hypersplenism indicates the existence of a relationship between these two organs. It has been suggested that humoral factors produced in the spleen stimulate bone marrow hematopoiesis (11). In contrast to the increased mitogen response of cells from splenectomized mice observed in the present work, the unaltered mitogen response of cells from mice inoculated with splenocytes indicates that this immune function was not altered by the procedure.

We think that the described animal model, consisting of inducing hypersplenism by intraperitoneal inoculation of syngeneic splenic cells, has several advantages over previous methods, especially the method of achieving hypersplenism by repeated injections of MC. Most studies with MC were performed with rats, whereas the method suggested here showed convincing results in mice. Although animals treated with MC in our study showed an enlarged spleen, they became sick, and two of the mice died, whereas those inoculated with splenocytes did not show any signs of distress. Moreover, MC-induced hypersplenism is not always accompanied by leukopenia (2, 12), and was even linked with hypoplasia of the bone marrow (4). The decrease in peripheral WBC counts accompanying the bone marrow hyperplasia in our model serves as a proof for the efficiency of our method.

1. Alwmark A, Bengmark S, Gullstrand P, Zoucas E. Hypersplenism—effect on hemostasis. An experimental study in the rat. Res Exp Med (Berl) 186:21–27, 1986.
2. Brabec V, Sebestic V. A study of experimental hypersplenism. Czech Med 7:107–116, 1984.
3. Fiala J, Viktora L, Jirasek A. Experimental hypersplenism in mice. Physiol Bohemoslov 25:167–172, 1976.

4. Zuckerman GB, Naughton BA, Gaito A, Preti RA, Gordon AS. The effect of methylcellulose on extrarenal erythropoietin production. Proc Soc Exp Bio. Med 176:197–202, 1984.
5. Sahin M, Tekin S, Akşoy F, Vatansev H, Seker M, Avunduk MC, Kartal A. The effect of splenic artery ligation in an experimental model of secondary hypersplenism. J R Coll Surg Edinb 45:148–152, 2000.
6. Weston SA, Parish CR. New fluorescent dyes for lymphocyte migration studies. Analysis by flow cytometry and fluorescence microscopy. J Immunol Meth 137:87–92, 1990.
7. Amorosi EL. Hypersplenism. Semin Hematol 2:249–285, 1965.
8. Palmer JG, Eichwald EJ, Cartwright GE, Wintrobe MM. The experimental production of splenomegaly, anemia and leukopenia in albino rats. Blood 8:72–80, 1953.
9. Leemans R, Harms G, Rijkers GT, Timens W. Spleen autotransplantation provides restoration of functional splenic lymphoid compartments and improves the humoral immune response to pneumococcal polysaccharide vaccine. Clin Exp Immunol 117:596–604, 1999.
10. Leemans R, Manson W, Snijder JAM, Smit JW, Klasen HJ, The TH, Timens W. Immune response capacity after human splenic autotransplantation: restoration of response to individual pneumococcal vaccine subtypes. Ann Surg 229:279–285, 1999.
11. Ruhrenstroth-Bauer G. The role of humoral splenic factors in the formation and release of blood cells. Semin Hematol 2:229–248, 1965.
12. Alwmark A, Bengmark S, Borjesson B, Gullstrand P. Treatment of hypersplenism. An experimental study in the rat. Eur Surg Res 14:322–332, 1982.