Local and systemic autonomic nervous effects on cell migration to the spleen

HEINER ROGAUSCH, DETLEV ZWINGMANN, MIRJAM TRUDEWIND, ADRIANA DEL REY, KARL-HEINZ VOIGT, AND HUGO BESEDOVSKY

Department of Immunophysiology, Institute of Physiology, Philipps-University, 35039 Marburg, Germany

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The function of the mammalian spleen is intensely intertwined with the detection of antigenic material circulating in blood, and splenectomy increases the risk of overwhelming infections or septic complications (3, 23). In contrast to other lymphoid organs, the spleen lacks significant afferent lymphatic vessels and blood flow represents the predominant route for the influx of cells and antigens. Two characteristic findings indicate the interdependence between splenic perfusion and cell uptake: 1) the flow per gram of tissue is 10 times higher than that of resting skeletal muscle and nearly as high as the blood flow of heart muscle, and 2) the rate of lymphocyte circulation into the spleen equals the total number of cells flowing into all other lymphatic and nonlymphatic organs (15, 16, 27).

It is remarkable that a strong interdependence between cell and blood supply coexists with a dense splenic noradrenergic innervation. If the content of norepinephrine (NE) is taken as reflection of the degree of noradrenergic sympathetic innervation, the spleen belongs to the most densely sympathetically innervated organs (5, 12), and the NE turnover rate is four to six times higher than that of the liver or lung. Most splenic noradrenergic nerve fibers have vasoconstrictor function and reduce blood flow. Therefore, the high splenic perfusion rate observed under basal conditions and during immune responses is surprising, but it can be explained by our laboratory’s previous observations that locally released cytokines, such as interleukin (IL)-1β, exert a tonic inhibition on the noradrenergic vasoconstrictor tonus (18). An increase in splenic blood flow mediated by a cytokine-inhibited NE release by sympathetic nerves may be a main mechanism influencing lymphoid cell uptake from the circulating cell pool (19). In addition, it is expected that the special morphological structure of the spleen guides cells and antigenic material from the circulation into the resident pool.

The analysis of how lymphocytes enter into splenic cell compartments suggests local regulatory influences either during the phase of cell uptake or during homing into specific areas (2, 26). However, these local mechanisms will still depend on the supply of lymphoid cells by blood circulation and on the particular structure of the spleen. Until now, the role of hemodynamic forces that determine splenic perfusion and cell uptake in this organ was not systematically investigated in vivo, and it is not known whether adhesion molecules can interfere or even override noradrenergic influences on splenic perfusion and blood cell supply. The experiments reported here tested the hypothesis that noradrenergic regulation of vascular blood flow plays a significant, but not exclusive, role for the splenic extraction of immune cells from the circulating pool.

We studied the influence of increased splenic vascular perfusion induced by either local denervation or general sympathectomy on lymphoid cell uptake by the

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spleen of normal and endotoxin-stimulated rats. The results obtained show that, although both procedures result in comparable increases in splenic blood perfusion, only local denervation produces a net increased influx of lymphoid cells into the spleen.

METHODS

General Procedures

Male inbred Wistar-Koyoto rats (300–350 g body wt) were housed in single cages and kept at a 12:12-h day-night cycle with water and standard pellets available ad libitum. Surgery was performed while the animals were under general anesthesia with pentobarbital sodium (60 mg/kg Narcoren). Animals were placed during surgery and experiments on a heating plate regulated to maintain core temperature between 36.5 and 37°C.

Determinations of Blood Flow

The procedure for the determination of organ blood flow with the microsphere technique is similar to standard, previously reported techniques (14, 21). Briefly, fluorescent-dye labeled polystyrene microspheres [excitation/emission 450/480 nm, diameter 15.5 μm (± 2%); Molecular Probes (MoBiTec, Göttingen, Germany)] were injected at 0.2 ml/min into the left ventricle at a dose of 450,000 spheres per animal. Simultaneously, a reference probe (Q̇ \text{ref}) was withdrawn from the abdominal aorta at the same flow velocity through the femoral artery, and blood flow in spleen, liver, heart, and skeletal muscle (Q̇ \text{i}) was determined by using the following equation

\[ Q_{i} = 100 \cdot \frac{Q_{\text{ref}}(I_{i}/I_{\text{ref}} \cdot W_{i})}{W_{i}} \]

where \( I_{i} \) and \( I_{\text{ref}} \) are number of spheres per tissue or reference probe, respectively, and \( W_{i} \) is weight of the tissue.

The heart minute volume was determined with an ultrasonic flow probe (model T206, flowmeter, Transonic Systems, Ithaca, NY) in animals that were not used for organ perfusion and cell uptake studies. The flow probe was placed around the thoracic aorta, and, after closure of the the chest and stabilization of blood gases and peripheral arterial resistance, blood flow was digitally registered at 80-kHz real-time display throughput (Dataga Instruments, Akron, OH).

In all animals, arterial blood pressure was measured via a catheter implanted in the femoral artery combined with a Statham pressure transducer; arterial Po2 and PCO2 were measured by electrochemical detection (Gas Check AVL, Bad Homburg), and core temperature was monitored with a thermistor probe in the abdominal cavity.

Labeling Procedure for Splenocytes

Splenocyte suspensions were prepared from inbred donor rats and labeled under sterile conditions with a PKH fluorescent cell linker kit (107 cells/ml in a 2 μM staining solution for 2 min; excitation/emission of PKH 551/567 nm; Sigma Chemical, St. Louis, MO). These cell suspensions were used because they mainly consist of lymphocytes; they need a minimum of purification steps. Before injection into the recipient, the number of dead cells was determined with trypan blue; the suspensions normally contained 6–10% dead cells; suspensions with <15% dead cells were also used. Cells (108 per kg body wt) were infused into the left ventricle over a period of 2 min in a volume of 1 ml, and the catheter was subsequently rinsed with 0.5 ml saline.

Evaluation of Splenic Cell Uptake

A piece of spleen was used to evaluate the uptake of labeled cells and the amount of trapped fluorescent spheres. The number of labeled cells per 100,000 splenocytes was determined from cyto centrifuge preparations of recipient spleen cell suspensions by using a fluorescent microscope. Because this step is critical for subsequent calculations, determinations were performed in parallel by two independent observers. Twelve cyto centrifuge preparations were evaluated from each cell suspension. Uptake of fluorescent cells was expressed either as the percentage of the number of injected cells, or per spleen or per 100,000 splenocytes.

Determination of IL-1β in the Spleen

Approximately one-third of the spleen was sonicated (20 s, 20 strokes, in ice-cold water), the homogenate was centrifuged (10 min, 20,000 g, 4°C), and IL-1β concentration in the supernatant was determined by using a commercially available kit for determination of rat IL-1β (Endogen, Woburn, MA).

Experiments

Control group. The uptake of fluorescent cells under baseline conditions was evaluated 15 min, 6 h, and 24 h after the injection of labeled cells (6 animals per time point). Blood flow was measured in the same animals 15 min or 6 h after cell injection, and the infusion of microspheres started 3 min before the spleen was removed.

Effect of vasodilatation induced by cutting the splenic nerve. The effect of local vasodilatation on fluorescent cell uptake into the spleen was evaluated by surgically interrupting splenic sympathetic nervous supply 5 days before the experiments were started as described previously (18). At this time, animals were recovered from the operation, as shown by their normal weight gain and corticosterone blood levels (see Table 1).

Successful denervation was documented by a 90% reduction of splenic NE content as evaluated by HPLC. The number of labeled cells that were sequestrated by the spleen and flow values were determined at the same times mentioned above. Sham-operated animals that were injected in parallel

### Table 1. Cardiovascular parameters in animals with intact splenic innervation (control), surgically denervated spleen (local denervation), or general depletion of vesicular noradrenergic stores (NE depletion)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Local Denervation</th>
<th>NE Depletion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arterial blood pressure, mmHg</td>
<td>103.5 ± 3.0</td>
<td>103.0 ± 3.2</td>
<td>90.2 ± 2.8*</td>
</tr>
<tr>
<td>Heart minute volume, ml/min</td>
<td>50.8 ± 3.4</td>
<td>55.3 ± 2.2</td>
<td>87.0 ± 2.7†</td>
</tr>
<tr>
<td>Arterial hemoglobin, g/dl</td>
<td>14.21 ± 0.33</td>
<td>13.92 ± 0.33</td>
<td>13.2 ± 0.24</td>
</tr>
<tr>
<td>Arterial Po2, Torr</td>
<td>95.8 ± 1.3</td>
<td>96.5 ± 0.6</td>
<td>95.2 ± 1.0</td>
</tr>
<tr>
<td>Arterial PCO2, Torr</td>
<td>40.3 ± 0.8</td>
<td>40.2 ± 0.8</td>
<td>40.7 ± 0.7</td>
</tr>
<tr>
<td>Splenic NE content, ng/g</td>
<td>412.7 ± 26.2</td>
<td>138.3 ± 6.6†</td>
<td>Not detectable†</td>
</tr>
<tr>
<td>Renal NE content, ng/g</td>
<td>127.7 ± 9.1</td>
<td>128.0 ± 8.7</td>
<td>7.1 ± 5.9†</td>
</tr>
<tr>
<td>Corticosterone in blood plasma, μg/dl</td>
<td>2.4 ± 0.9</td>
<td>2.6 ± 0.4</td>
<td>1.9 ± 1.0</td>
</tr>
</tbody>
</table>

Values are means ± SE for 6 animals per group. NE, norepinephrine. *P < 0.05; †P < 0.001 compared with control.
Effect of vasodilatation by depletion of sympathetic noradrenergic stores. Because, under clinical conditions, pharmacological interventions can affect not only splenic but also sympathetic nerve endings in other organs, general noradrenergic transmission was interrupted by depleting noradrenergic stores with reserpine (10 mg/kg ip; 24 h before the experiment). Six animals treated with reserpine and six animals injected with the vehicle alone were studied in parallel at the same points of time as in the other studies.

Effect of endotoxin on splenic cell uptake. Endotoxin [lipopolysaccharide (LPS)] from Escherichia coli (026:B6; TCA extract; Sigma Chemical; 10 μg/kg body wt) was dissolved in isotonic sodium chloride solution and injected into the tail artery in awake animals. Six hours later, labeled cells were injected as described above, and cell uptake was determined at the same time intervals as in the other experimental groups.

Statistics

The data were compared by ANOVA and Scheffé’s test. All values are reported as means ± SE from six rats, and statistical significance was set at P < 0.05.

RESULTS

Relation Between Splenic Blood Flow and Cell Uptake

Blood flow of the spleen was measured under basal conditions and after interruption of noradrenergic innervation. Basal flow was 73.5 ± 8.65 ml/100 g·min⁻¹. This value approached myocardial perfusion (91 ± 8.00 ml/100 g·min⁻¹) and was nearly 20 times higher than the perfusion of skeletal muscle at rest (3.8 ± 0.6 ml/100 g·min⁻¹), as measured in the same animal and under the same experimental conditions. Because the spleen has a high basal blood flow value, it was important to determine whether it can be increased after interruption of the noradrenergic innervation. The results showed that despite high resting flow, the ablation of the noradrenergic innervation led to a significant increase: 276.7 ± 10.6 ml/100 g·min⁻¹ after local surgical denervation (P < 0.001), and 265 ± 10.6 ml/100 g·min⁻¹ after depletion of noradrenergic presynaptic stores (P < 0.001; Fig. 1). The increase in flow indicates that the noradrenergic sympathetic innervation of the spleen has profound influence on establishing the level of splenic perfusion. Sham-operated and untreated animals exhibited no significant differences of splenic blood flow.

Next, we wanted to know whether an increased local perfusion favors the accumulation of injected cells in the spleen. Both 15 min and 6 h after the injection of labeled cells, a higher sequestration of injected cells (Fig. 2A), higher number of cells retained per spleen (Fig. 2B), and higher number of cells retained per 100,000 splenocytes (Fig. 2C) were observed in the denervated spleen compared with the controls. Even after 24 h, more cells accumulated in the denervated spleen compared with the sympathetically innervated organ. These results suggest the possibility of parallel changes between spleen perfusion and cell uptake, which was corroborated by the experiments described below.

Cell Sequestration into the Spleen Is Favored by an Increased Splenic Perfusion Induced by Bacterial Endotoxin

Studies in which LPS was used were included because this cytokine raises blood flow and can favor the adhesion of leukocytes on endothelial cells. If adhesion is additive to the effect of perfusion, a higher cell uptake into the spleen, which exceeds the effect of increased perfusion, would be expected. However, Fig. 3 indicates parallel changes between the level of splenic perfusion and cell uptake. A positive correlation between local perfusion and the degree of cell extraction from circulation is described by a second-order polynomial equation (y = 1 × 10⁻⁵x² + 0.008x + 0.074, R² = 0.74; Fig. 3).

Because, as our laboratory reported before (19), IL-1β is the main mediator of the increase in splenic blood flow induced by LPS, the production of this cytokine in the spleen of rats subject to either local or general denervation was evaluated. LPS administration significantly increased IL-1β in the spleen, but the increase was comparable in control or local and systemic denervated rats (IL-1β ng/spleen: none + vehicle = 2.57 ± 0.35; none + LPS 28.9 ± 6.32; sham operated + vehicle = 5.7 ± 0.55; sham + LPS = 27.9 ± 3.85; local splenic denervated + LPS = 19.37 ± 3.19; system-
ically denervated + LPS = 24.1 5.8; 4–6 rats per group). The IL-1β content in the spleen of rats from all groups that received LPS differed significantly from the controls (P < 0.05); the different denervation procedures did not significantly affect LPS-induced IL-1β content.

Systemic Vasodilatation Interferes with Local Cell Supply

The vasodilatation observed in the spleen after systemic depletion of noradrenergic stores was similar to the effect of local denervation (Fig. 1). Both procedures induced comparable reductions of the splenic NE content, but only systemic denervation affected the content of the neurotransmitter in other organs, such as the kidney (Table 1). Accordingly, although local denervation increased blood flow only in the spleen, the abrogation of general noradrenergic vasoconstrictor tone induced higher blood flow also in other sympathetically controlled organs. This effect was followed by a significant increase of the heart minute volume to maintain arterial blood pressure at normal levels (Table 1).

The redistribution of the heart minute volume between peripheral organs included increased blood flow in large parenchymatous organs. For example, liver blood flow rose from 3.7 ± 0.5 to 10.1 ± 1.4 ml/min, i.e., by 6.4 ml/min (Fig. 4); skeletal muscle flow increased from 3.8 ± 0.6 to 45.0 ± 11.4 ml·100 g⁻¹·min⁻¹. As already shown in Fig. 1, systemic NE depletion also resulted in an increase of splenic blood flow of ~200%, but this increase was low (only 1.5 ml/min) compared with that observed in other organs when expressed in
Our results demonstrate that the uptake of circulating lymphocytes by the spleen is favored by a selective, local increase in splenic perfusion induced by splenic denervation and by the bacterial endotoxin LPS. Furthermore, we report here that general vasodilatation induces opposite effects on immune cell uptake by the spleen. With respect to LPS, it should be mentioned that the increase in blood perfusion induced by doses of the endotoxin that do not cause shock is restricted to the spleen without affecting other lymphoid organs, and this effect is mediated by the capacity of locally released IL-1β to inhibit the sympathetic tonus (19). Because cytokines released after endotoxin administration can promote the expression of adhesion molecules on endothelial cells, it can be expected that LPS stimulates cell uptake by that mechanism (22, 28). The spleen has no high endothelial venules, but adhesion molecules, such as integrins and selectins, are expressed in the extracellular matrix of the splenic meshwork, and they may be upregulated by cytokines induced by LPS and contribute to the high capacity of the spleen to sequestrate cells from the blood (15, 16). IL-1β is equally produced in the spleen in locally and systemically denervated or normal innervated spleens, and differences in cell uptake cannot be explained by differences in IL-1β production. However, the results reported here, although corroborating that splenic cell uptake is favored by LPS, indicate that hemodynamic influences also play a relevant role in the capacity of the spleen to uptake circulating cells.

We studied splenic cell uptake instead of determining the circulation half-time of injected cells. This approach was chosen because the number of circulating lymphoid cells depends on a large number of factors, including redistribution of cells between marginating, adhering, or emigrating pools. Furthermore, the measurement of half-times of circulating cells provides no information about where cells are homing, whereas the determination of cells accumulating in the different organs gives a better indication of local cell uptake. Because it has been shown in vivo that removal of sympathetic noradrenergic transmission has no measurable influence on the size of splenic cell compart-

**DISCUSSION**

Our results demonstrate that the uptake of circulating lymphocytes by the spleen is favored by a selective, local increase in splenic perfusion induced by splenic denervation and by the bacterial endotoxin LPS. Furthermore, we report here that general vasodilatation
ments, the rate of cell proliferation, or apoptosis (6), it
is unlikely that these processes would influence the
number of cells uptaken by the spleen. The interrupt-
tion of the noradrenergic transmission is frequently
used to detect autonomic nervous influences on im-
mune functions (for reviews, see Refs. 5, 16). The
nearly complete abrogation of the noradrenergic vaso-
constrictor influence, most likely mediated by inhibi-
tion of NE release after administration of LPS to ani-
mals with an intact innervation, results in an increase
in blood flow that is close to that induced by sympa-
thectomy (19, 20). Thus the effect of denervation can
be considered as a situation reflecting what would occur
under more physiological conditions. An increase in
blood flow similar to that caused by denervation is also
noticed during local inflammatory processes and in the
local hyperemia that precedes specific immune re-
sponses. Our results indicate that, under these condi-
tions, the locally increased blood flow would direct the
cells to the sites of immune defense.

The fact that splenic blood flow increases in a com-
parable magnitude both after local and general inter-
ruption of noradrenergic sympathetic transmission
and after LPS, but that only local denervation or LPS
administration results in increased splenic cell uptake,
may be related to the particular function of the spleen,
which can be considered as a filter inserted in the
arterial circulation. This view is briefly discussed be-
low.

There are still controversial results about adhesion
and recognition of circulating leukocytes at the endo-
thelial lining of blood vessels and about the effects of
NE and sympathetic nerves on these processes (4, 10,
11, 17, 24, 26). An argument against cell sorting at the
site of entrance into the spleen is the finding that
memory cells and cytotoxic effector T lymphocytes are
migrating in comparable numbers into the spleen,
whereas they are differentially extracted from the cir-
culating pool into other lymphoid organs (25).

Our results indicate that the absolute level of splenic
perfusion (ml/min) is not the only variable determining
the number of cells that are trapped by the spleen,
because it also depends on the distribution of blood flow
within peripheral organs. We showed that normal
spleen perfusion represents ~2% of the total heart
minute volume and that it increases to ~4% after local
denervation. After general interruption of sympathetic
noradrenergic transmission, the spleen receives ~2%
of the heart minute volume, i.e., not more than when
sympathetic innervation is intact. This may explain
why during general vasodilatation not more cells are
taken up by the spleen than under control conditions,
despite the fact that splenic perfusion is higher than
under basal conditions. The increased blood flow in
large parenchymatous organs may diminish the
chance of circulating lymphocytes to contact the
splenic meshwork. After contacting endothelial cells,
only 4–6 of 10 lymphocytes emigrate from the blood-
stream (9), and prolonged circulation through extras-
plenic pathways during general vasodilatation may
further reduce splenic cell uptake. Such a prolonged
extrasplenic circulation might be also relevant for the
dynamics of uptake of particulate antigenic material.

The present data may have clinical implications.
Vasodilating drugs and physiological or pathophysio-
logical conditions leading to general vasodilatation
would reduce splenic uptake of cells or circulating
antigenic material and therefore interfere with the
protective function of the spleen. For example, during
physical stress, blood flow decreases preferentially in
the spleen because it is one of the most densely nor-
adrenergically innervated peripheral organs, whereas
other organs are not affected so much or are even more
perfused, such as skeletal muscle at work (7, 8). The
present results predict that, under this condition, the
contact of circulating cells with splenic tissue is re-
duced. Such interpretation is supported by recent ex-
eriments showing that after prolonged α-adrenergic
stimulation, the number of leukocytes increases in
blood circulation and decreases in the spleen (24).

It can be concluded from our results that after the
redistribution of the heart minute volume during in-
tense muscular work, i.e., in a condition where NE
levels increase and skeletal muscle vasodilatation pre-
vails, not only cell uptake, but also trapping of circu-
lating antigenic material in the spleen, is reduced. This
condition may contribute to the impairment of immune
defense observed after exhausting physical training, a
situation during which the blood flow of the muscle is
increased because of high metabolic demands but
splenic blood flow is reduced because of sympathetic
activation (13).

In conclusion, our results stress the relevance of
hemodynamic forces controlled by the sympathetic ner-
svous system for cell and antigen uptake by the spleen.
Immune processes that cause only a local increase in
blood flow would favor splenic cell uptake and immune
defense. On the contrary, general vasodilatation would
interfere with the capacity of the spleen to extract cells
from the circulation and thus interfere with splenic
immune functions.

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