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THE ROLE OF THE SPLEEN IN REGULATION OF EXTRACELLULAR FLUID IN THE RAT

BY

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A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled THE ROLE OF THE SPLEEN IN REGULATION OF EXTRACELLULAR FLUID IN THE RAT submitted by AREANA YEN CHEN in partial fulfillment of the requirements for the degree of MASTER OF SCIENCES in EXPERIMENTAL MEDICINE.

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To my family and fiancé, for all their love and support.

ABSTRACT

Fluid extravasation from the splenic circulation was investigated by implanting ultrasonic flow probes around the splenic vessels of rats. Splenic blood flow and splenic arterio-venous difference were lowest at the time of surgery and then dramatically increased during recovery. When sodium pentobarbital (20mg/kg, iv) was given to conscious, recovered rat, there was a transient fall in splenic blood flow. However, although the arterio-venous difference tended to fall, it nevertheless remained significant throughout the experimental period. In response to hemorrhage (1.5% body weight), both splenic blood flow and arterio-venous difference were markedly decreased. It is concluded that:

- Basal splenic blood flow is higher in the fully recovered and conscious rat than in acute preparation;
- Up to 30% of fluid is extracted from circulating blood in spleen;
- Anesthesia reduces splenic blood flow and fluid efflux, but not to the extent seen during surgery;
- Hemorrhage depresses both splenic blood flow and the arterio-venous difference.

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
ANF	Atrial natriuretic factor
BW	Body weight
GFR	Glomerular filtration rate
MAP	Mean arterial pressure
PALS	Periarterial lymphatic sheath
RBC	Red blood cell
RP	Red pulp
WP	White pulp

CHAPTER 1

INTRODUCTION TO THE THESIS

AND

GENERAL PHYSIOLOGICAL FUNCTION OF THE SPLEEN

CHAPTER 1

INTRODUCTION

Researchers have long tried to understand the structure and function of the spleen. In the second century AD, Galen described the human spleen as an enigmatic organ. Early in the 17th century, when Malpighi studied the white pulp (WP), red pulp (RP) and splenic lymph follicles under the microscope, there was uncertainty as to whether the spleen had a secretory function. The role of the spleen in the elaboration and "purification" of the circulation blood was later investigated and described by van Leeuwenhoeck. In 1722, Goulsonian totally rejected the concept of the spleen as a secretory organ, but believed that the spleen acted as a controller of blood volume. This idea was widely supported by Cooper, Winslow, Heister and Hodgkin, and was modified by Gray in 1854. In Gray's *Textbook of Anatomy*, splenic function was thus described-"to regulate the quantity and quality of the blood". Between 1923-1932, Barcroft realized that this controlling mechanism in the spleen was very complicated and could be influenced by various factors. Again, it was many years before researchers finally recognized that the human spleen had very limited physiological storage function for red blood cells (Bowdler, 1990). In the second half of this century, 1970s Nieuwenhuis, Ford, and Keuning performed immunological function studies on the rat spleen (Han, Van Krieken & te Velde, 1992), and the fine structure and detailed description of the rat spleen was published (Veerman, 1975). Despite these careful studies, it was believed for some time

that there were no deep lymphatics in the murine spleen (Maximow & Bloom, 1941, Yoffee & Courtice, 1970). The existence of splenic deep lymphatics was just recently verified (Pellas & Weiss, 1990; Koshikawa, Asai & Iijima, 1984).

The human spleen (Gr: splen, L: lien) is about the size and shape of a clenched fist. It is a large, soft and friable vascular lymphatic organ lying superior and posterior to the cardiac end of the stomach at the left level of the 9th, 10th and 11th ribs, with its long axis parallel to them. The size of the spleen varies from 12 - 15 cm in length, 4 - 8 cm in width and 3 - 4 cm in thickness. The average splenic weight in the adult ranges from 0.2 - 0.25 % proportion of body weight, which is about 150 - 180 g in an adult male, and surpasses that of the adult female by 40 g. The spleen gradually loses its total weight to 60 g in the seventh and eighth decades. Splenic size differences may reflect the physiological condition of the subject. Resting splenic blood flow in all species has been reported to lie between 40 - 100 ml/min per 100 g of tissue corresponding to 1 - 10 % of the cardiac output. Also, the human spleen stores about 30% of the total platelet mass of the body. Average red cell content of the spleen is about 30 - 50 ml (Peter & Peter, 1990; Davies & Withrington, 1973; Groom & Schmidt 1990).

As the greatest aggregation of lymphoid tissue in the body, the spleen constitutes the only lymphoid organ specialized for the filtration of blood. All spherocytes, abnormal red blood cells and microorganisms are removed and destroyed in the spleen. In addition

to the abundance of lymphocytes, macrophages and white blood cells, splenic immunological defense function is well established. The spleen also serves as a reservoir for platelets, the release of platelets during hemorrhage being critical for stopping further blood loss.

Despite all these previous studies, there was still much confusion surrounding the histology and function of the spleen, because many of these splenic functions had not been systematically observed under normal conditions. Quite apart from a blood storage function, it is reasonable to believe that the spleen plays an important role in the regulation of the vascular circulation. Unfortunately, there had been very little information published regarding splenic hemodynamic changes under physiological conditions. Also, the conflicting evidence regarding the existence of splenic deep lymphatics needed to be further investigated. The physiological and/or pathological role played by the spleen in regulating extracellular fluid volume had not been studied until experiments showed splenic venous hematocrit to be consistently higher than splenic arterial hematocrit (Kaufman & Deng, 1993). Further evidence suggested that, in response to expansion of the intravascular space, there was increased intrasplenic filtration of plasma out of the blood and into the lymphatic system (Kaufman & Deng, 1993). In order to further investigate that there is red cell-free fluid being extracted from the circulation, and to confirm that the high splenic venous hematocrit was not the result of expulsion of extra red blood cells (RBC) from the spleen, a comparison of splenic

afferent and efferent blood flow was made. It was anticipated that this would provide quantitatively proof of the existence of fluid efflux in the spleen.

In response to hypervolemia, there are increases in atrial pressure, end diastolic ventricular pressure, cardiac output and arterial blood pressure. These changes stimulate the cardiopulmonary and arterial baroreceptors which initiate movement of fluid and electrolytes out of the intravascular compartment. It is also well established that urine output increases under such conditions. By these mechanisms, the body achieves both redistribution and elimination of excess extracellular fluid (Brenner et. al, 1990).

Plasma atrial natriuretic factor (ANF) levels also rise due to the increase of atrial pressure. ANF is a hormone secreted mainly by atrial myocytes in response to local wall stretch and atrial pressure (i.e. increased intravascular volume). ANF produces natriuresis, diuresis, as well as diminished arterial blood pressure. In the vasculature, ANF reduces peripheral vascular resistance through peripheral vasodilatation. Plasma ANF contributes largely to changes in renal hemodynamics by an increase in glomerular filtration rate (GFR) and filtration fraction, by inhibition of net Na^+ reabsorption and water reabsorption, and by suppression of renin secretion in the macula densa. ANF also facilitates transudation of intravascular fluid to the interstitium. Furthermore, both aldosterone and adrenocorticotropic hormone (ACTH) secretion are inhibited by ANF so that renal salt and water reabsorption decrease and urine output increases. With all these

combined actions of ANF on the vasculature, kidneys and adrenals, both systemic blood pressure and intravascular volume decrease (Brenner et al, 1990).

Evidence has shown that, during intrasplenic infusion of ANF, the splenic venous hematocrit gradually increases in a dose dependent manner (Kaufman & Deng, 1992). Moreover, splenectomy abolishes the non-renal effects of ANF on both the rise in hematocrit and the fall in plasma volume (Kaufman, 1992). Reports have shown that, in response to volume loading or atrial distention, there is also a relative increase in the hematocrit of blood draining from the spleen. Furthermore, after injection of Evan's Blue into the splenic artery of rats, the dye rapidly appears in the lymphatic fluid draining through the splenic lymphatic duct (Kaufman & Deng, 1993). A dramatic increase in both transcapillary fluid flux and thoracic duct lymph flow in response to saline loading has also been shown by Vanlenzuela et al in 1989. Moreover, the periarterial lymphoid sheath (PALS) of the splenic white pulp (WP) is reported to act as a central station of the extravascular pathway which is connected to the deep lymphatics and the marginal zone. High lymph flow to the deep lymphatics from the red pulp (RP) has been found through this periarterial lymphatic sheath (Koshikawa, Asai & Iijma, 1984). All these findings suggested that the spleen might play a very important role in the regulatory control of fluid extraction from the circulation (Hartwig & Hartwig, 1985; Opdyke, 1970; Stock et al, 1983). It was therefore proposed that *there is intrasplenic filtration of red cell-free fluid from the blood into lymphatic reservoirs, and that this fluid efflux is subject to regulatory control.* However, the mechanisms underlying this fluid redistribution were

not known. Moreover, it had not even been *directly* proven that fluid is extracted from the splenic circulation. The presently reported studies addressed this concept, and further investigated the mechanisms underlying intrasplenic filtration.

In humans, the splenic capsule is nonmuscular and splenic function as a blood reservoir is considerably less developed than in other animals such as the horse and the dog (Reilly, 1985). It is well known that the spleen is the largest and only lymphoid organ specialized for the filtration of blood. However, the mechanisms underlying these functions are not well understood (Groom & Schmidt, 1990). The rat is an ideal model in which to study this phenomenon since in this species, like in humans, the spleen has very limited blood storage capacity (Reilly, 1985). Thus measurements of splenic blood flow are not confounded by expulsion of high hematocrit blood (Davies & Withrington, 1973). Furthermore, rats have the same elaborate sinusoidal network and fast blood flow pathways as humans, and red blood cell washout tests show that the rat's spleen is qualitatively similar to humans (Groom & Schmidt, 1990). It has been reported that blood flow to the spleen is extremely high (Gross, 1985), in excess of 6% of cardiac output in the rat (Groom & Schmidt, 1990). Therefore, *the spleen has great potential to effect changes in circulating blood volume.*

The path taken by the blood through the spleen depends on the state of volemia of the animal. In the relaxed euvolemia control spleen, 90 - 93% of the inflow blood travels to the splenic vein *via* the fast pathways with a washout half-time of 30 seconds, while

only 7 - 10% of the total blood flow travels through the slow and intermediate pathways of the reticular meshwork for filtration in 8.4 - 54 minutes (Groom & Schmidt, 1990; Groom & Song, 1971; Stock et al, 1983). Under conditions of hypovolemia, the total blood passing through fast pathways containing arterial/venous shunts increases from 90 to 98.7%; the washout half-time remains between 20-30 seconds (Levesque & Groom, 1981; Groom & Song, 1971). Splenic filtration of blood also decreases during hypovolemia and anemia since most red blood cells are shunted around the red pulp (Stock et al, 1983). In contrast, in hypervolemia, the blood takes a slow, tortuous path through the red pulp of the spleen (Levesque & Groom, 1980). It is of interest therefore to note that both volume loading and stimulation of the atrial volume receptors with an intracardiac balloon have shown the increase of the fluid efflux from the splenic circulation (Choi & Kaufman, 1991).

It is proposed that, due to its extremely high blood flow (Gross, 1985) and great potential to effect changes in circulating blood volume, the spleen might be one of the important organs that regulates intravascular volume. After volume loading, the two surfaces of the mesentery enveloping the vascular arcade supplying the spleen have been observed to become separated by a clear gelatinous substance. It is believed that these are reservoirs where fluid, isooncotic to the plasma, may be stored (Chen & Kaufman, 1995). This brought about the first aim of the present study: to establish an animal model for *directly* measuring simultaneous splenic afferent arterial blood flow and splenic efferent

venous blood flow, so that not only the splenic circulation could be studied, but fluid efflux from the splenic circulation might also be measured.

Since virtually all previous experiments on splenic function had been done on anesthetized animals, and since it had been well known that anesthesia increases total peripheral resistance and disrupts reflex cardiovascular homeostasis (Matsukawa, Ninomiya & Nishiura, 1993; Berne & Levy, 1992), the experimental protocol was designed to cover the day of surgery as well as the recovery period up until day 10. In this way, the effect of anesthesia alone, without surgery, on splenic blood flow could be studied.

It is well known that, during hemorrhage, the principal homeostatic adjustments relate to the cardiovascular system. Mean arterial pressure and pulse pressure decrease during hemorrhage, thus reducing stimulation of the baroreceptors in the carotid sinuses and aortic arch. Reduction of vagal tone and enhancement of sympathetic tone increase heart rate and myocardial contractility to compensate for the hemorrhage. Also the increase in sympathoadrenal discharge and sympathetic activation produce arterial vasoconstriction and venoconstriction. Since certain vascular beds, such as the cutaneous, pulmonary and hepatic circulation are preferentially constricted, venous return to the heart is maintained (Berne & Levy, 1992).

It has been reported that during this sympathoexcitation period there is significant increase in splenic sympathetic nerve activity associated with unloading of the cardiopulmonary receptors and arterial baroreceptors. Although stimulation of arterial pressoreceptors has little effect on splenic nerve activity, the carotid baroreceptors have been shown to affect splenic dimensions in anesthetized cats (Maass-Moreno & Rothe, 1991). Furthermore, there are reports showing that changes in splenic vascular volume, mediated through vagal afferent nerves were faster and of greater magnitude than those in other nerve activities (Koyama et al, 1992). After the initial hemorrhage-induced sympathoexcitation, the progressive hemorrhagic hypotension induces sympathoinhibition where the *sustained* unloading of the cardiopulmonary receptors appear to further stimulate the cardiac vagal afferents. It is also known that, during hemorrhage, cardiac vagal afferents elicit greater and faster augmentation of splenic sympathetic activity than any other nerve activity, such as hepatic, renal, cardiac and adrenal sympathetic nerve activities (Koyama et al, 1992). Although *in vivo* experiments show that, in the contracted spleen, the flow of red cells and plasma might be confined entirely to the fast blood flow pathways during sympathoexcitation, sympathoinhibition might evoke different splenic circulatory homeostasis. Therefore, it is important to study the hemorrhage effect on the splenic blood flow, and to further investigate if there is any influence of the low pressure volume receptors on splenic circulatory function (Koyama et al, 1992).

A variety of reports have shown that sympathetic nerves and catecholamines produce contraction or spasm of lymphatics *in situ*. Norepinephrine increases both the tension (Mawhinney & Roddie, 1973) and the frequency of spontaneous contractions (Allen, McHale & Rooney, 1983) in segments of bovine lacteals. Epinephrine *in situ* has been reported to cause visible reductions in lymphatic vessel diameter (Florey, 1927). In addition, infusion of catecholamines or increased sympathetic discharge reduces transvascular efflux of fluid from the microvessels and, as a result, causes a decrease the formation and flow of lymph (Dabney, Buehn & Dobbins, 1988).

It is proposed that, in response to the hemorrhage, there is a decrease in the intrasplenic filtration of red cell-free, isooncotic fluid out of the blood and into lymphatic stores. Although it was well known that sympathetic tone is high at the time of surgery, a recent paper has shown that, in anesthetized rats, there are no signs of the sympathoexcitatory phase at all (Leskinen, Ruskoaho & Huttunen, 1994). Lymphatic pumping is also suppressed by anesthetic agents (Drake & Gabel, 1991). Furthermore, there is evidence showing that plasma vasopressin levels are elevated dramatically during invasive surgery (Corman & Geelen, 1992). All these responses would definitely interfere with splenic circulation and lymphatic function. Therefore, it was very important to establish a fully recovered unrestrained *conscious* hemorrhage animal model for direct investigation of the splenic circulatory mechanism and response to hemorrhage. By directly measuring splenic arterial and venous blood flow, splenic hemodynamic performances to hemorrhage could be observed. Thus, the important role of the spleen in

adjustment of cardiac performance and maintenance of circulatory homeostasis during hemorrhagic hypotension could be interpreted.

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CHAPTER 2

SPLENIC BLOOD FLOW

AND

FLUID EFFLUX FROM THE INTRAVASCULAR SPACE

This paper was submitted to *Journal of Physiology* in January 1995.

CHAPTER 2

SUMMARY

1. Previous evidence had suggested that fluid was extracted from the blood during its passage through the splenic circulation. In order to further investigate this phenomenon, flow probes were placed around the splenic artery and vein of rats. Splenic blood flow and mean arterial pressure (MAP) were measured during surgery, and 5 and 10 days later. On day 5, rats were administered sodium pentobarbitone I.V. to investigate the effect of anesthesia on splenic circulation.
2. Splenic arterial blood flow was lowest at the time of surgery. It had doubled by day 5, and remained stable thereafter at about 7 ml/min. Splenic venous blood flow also increased after surgery, but less so than the arterial flow, so that the arterio-venous difference had increased to 2.0 ± 0.4 ml/min by day 5. The arterio-venous difference remained quite constant during the 1 hr post-stabilization period.
3. In response to I.V. sodium pentobarbitone, there was a transient fall in splenic blood flow. There was no significant change in the arterio-venous difference of blood flow, although it tended to decrease.
4. In conclusion, we have shown that splenic blood flow is considerably higher than previously reported. We have also revealed a significant new path for fluid efflux from the intravascular space; 30% of fluid volume flowing into the spleen is removed from the circulating blood into the lymphatic system.

5. Although anesthesia modulates splenic blood flow, the changes are not as pronounced as those observed in the acute surgically prepared animal.

Index terms: Spleen, rat, blood volume, anesthesia, sodium pentobarbitone

INTRODUCTION

ANF causes a reduction in plasma volume that cannot be accounted for by urinary losses (De Bold, Borenstein, Veress & Sonnenberg, 1981). The report which showed that splenectomy abolishes this response (Kaufman, 1992), prompted this study to investigate whether the spleen might be a site of fluid efflux from the intravascular space. In fact, in response to volume loading or atrial distention, there was a relative increase in the hematocrit of the splenic venous effluent (Kaufman & Deng, 1993). Plasma protein concentration did not change as the blood passed through the splenic circulation. Further experiments also revealed that clear fluid was seen to drain down the splenic lymphatic ducts and to accumulate in a gel-like matrix in the mesentery around the splenic vascular arcade; the protein concentration of this fluid was very close to that of the plasma. Thus, the hypothesis of this study is that, *in response to expansion of the intravascular space, there is increased intrasplenic filtration of red cell-free, isooncotic fluid out of the blood and into the lymphatic system.*

It could be argued that the increase in hematocrit of the splenic venous blood could conceivably have been caused by the addition of enriched blood from intrasplenic stores, although this capacity is extremely limited in the rat (Reilly, 1985). In order to definitively show that this was not the case, and that fluid was *removed* from the splenic blood, not added to it, this study was designed to directly measure splenic arterial and venous blood flow with transit time flow probes. Since the previous experiments were

done acutely in anaesthetized rats (Kaufman & Deng, 1993), and since there were questions at that time as to whether high sympathetic tone might have altered splenic circulation, this study determined also to investigate the effect of surgery and anesthesia on splenic blood flow and fluid efflux.

MATERIALS AND METHODS

The experiments described in this paper were examined by the local Animal Welfare Committee, and found to be in compliance with the guidelines issued by the Canada Council on Animal Care.

ANIMALS:

Male Long Evans rats weighing between 450 and 600g (retired breeders) were obtained from Charles River (St. Foy, Quebec). They were held in the University Animal Facility for at least 1 wk before any surgical or experimental procedures were started. The animal room was temperature and humidity controlled, with a 12:12 light/dark cycle. The rats were maintained on a 0.28% sodium diet (Purina) and water *ad libitum*.

DRUGS:

All surgical procedures were carried out under sodium pentobarbitone anesthesia (60 mg/kg body weight, I.P.) plus atropine (0.12 mg/kg body weight, SC). Dihydrostreptomycin (Derapen-C, 0.22 ml, I.M.) was given pre-operatively. In order to

minimize problems resulting from damage to the pancreatic tissue during the surgery, the proteinase inhibitor aprotinin was administered (Trasylol, 0.3 ml, I.V.). After the flow probes were implanted, a few drops of Xylocaine (Lidocaine 10 mg/ml) were applied to the area to dilate the splenic vessels. Following surgery, an analgesic Buprenorphine Hydrochloride (0.02 mg/kg body weight, I.M.) was given every 8 hours for 2 days.

SURGERY:

Cannulation of femoral artery and vein: Silastic (Dow, 0.51 mm ID, 0.94 mm OD) and Micro-Renethane (Braintree, 0.30 mm I.D., 0.64 mm O.D.) cannulae were placed respectively in the femoral vein and artery. The femoral arterial cannula was used for monitoring blood pressure and the femoral venous cannula was used for loading isotonic saline or injecting the test drug. The cannulae were exteriorized at the nape of the neck via stainless steel tubing, and filled with approximately 200 µl of heparinized saline (10,000 units/l) to maintain patency.

Implantation of blood flow probes : A midline laparotomy was made, extending up to the xiphisternum. The spleen was carefully cleared from its attachments to the stomach, and the stomach reflected up and out of the abdominal cavity. In order to ensure that the splenic artery and vein supplied and drained only the spleen, all the branching vessels, such as the short gastric vessels, and those running from the splenic vessels to the pancreas, stomach and other surrounding tissue, were ligated and divided (Figure 1). Great care was taken in clearing the pancreatic and fatty tissue from the splenic artery and

vein with wet Q-tips. The flow probes (Transonic transit time ultrasonic probes, R1), were glued into position on a sheet of Silastic, and placed under the splenic vascular arcade. The splenic artery and vein were slipped into the probe windows (Figure 2). The stomach was then replaced into position over the splenic vessels and probes, and the leads from the probes led subcutaneously to the back of the neck.

EXPERIMENTAL PROTOCOL:

The rats were deprived of food, but not water, for 24 hr after surgery in order to minimize secretion of digestive enzymes from the pancreas. The rats were then offered a high energy liquid diet (Ensure Plus) in addition to their regular chow and water. On days 4 and 9, the day before each experiment, the rats were placed in metabolism cages with food and water. (This facilitated access to the cannulae and probe leads.) The next day, the femoral arterial cannula was connected to the blood pressure transducer, and the femoral venous cannula to a syringe for injection of test substances or saline. The blood flow probes were connected to an ultrasonic, transit time blood flowmeter (Transonic, NY). The rats were maintained unrestrained in their metabolism cages throughout the experimental period. Splenic afferent and efferent blood flows, and blood pressure, were recorded simultaneously.

Splenic blood flow was measured in all animals at the time of surgery, and at day 5 (n=34). Fifteen of the rats were retested at day 10 post-surgery. During the test period, the rats were kept in a quiet room, and observed through a window. After one hour of

stabilization, basal measurement of splenic arterial and venous blood flows, and arterial blood pressure, were recorded for 10 min. On day 5, blood flow measurements were continued for an additional 60 min.

The effect of pentobarbitone anesthesia on splenic blood flow was also measured. On day 5, the conscious rats were randomly divided into two groups: (a) control group - the rats were administered saline (0.3 ml, I.V., n=11), and (b) anesthetic group - the rats were administered sodium pentobarbitone (20 mg/kg I.V., n=10). Different animals were used for each group i.e. the animals did not act as their own controls. Following administration of either anesthetic or saline at the end of the 1hr stabilization period, splenic afferent and efferent blood flow, and arterial blood pressure were recorded every 2 min for 10 min and then at every 5 min for 30 min.

STATISTICAL ANALYSIS:

The statistical significance of the differences between splenic afferent and efferent blood flow was assessed by Student's t-test for paired data. The significance of the changes in blood flow over the recovery time (days 5 and 10) were assessed by ANOVA, followed by Student-Newman-Keuls test for multiple comparisons. The significance of the changes in splenic blood flow and mean arterial blood pressure caused by sodium pentobarbital was tested using Student's t-test for unpaired data. The level of statistical significance was defined at $P< 0.05$. Data are expressed at means $\pm$ SEM.

RESULTS

Mean splenic arterial blood flow was lowest at the time of surgery (anesthetized animals). It had doubled by day 5, and remained stable thereafter at about 7 ml/min (Figure 3). Splenic venous blood flow also increased after surgery, but to a lesser extent, so that the arterio-venous difference steadily increased until, at day 10, it was 2.5 $\pm$ 0.5 ml/min (n=15) (Figure 4). Resting mean arterial pressure in the conscious rats did not change during the period from 5 to 10 days (97 $\pm$ 3 mm Hg on day 5; 98 $\pm$ 5 mm Hg on day 10)

On any given day, arterial and venous blood flows tended to fall during the experimental period. However, the arterio-venous difference remained quite constant (Figure 5).

In response to I.V. sodium pentobarbitone, there was a significant, but transient, fall in arterial and venous blood flow to the spleen (Figures 6 and 7). The arterio-venous difference also tended to decrease but did not reach significance compared with the control group (Figure 8). Mean arterial pressure fell when the anaesthetic was given, and remained significantly depressed for the entire experimental period (Figure 9).

DISCUSSION

Blood flow to the spleen had been previously reported to be about 2 ml/min (Flaim & Zelis, 1982). This was considered to be extremely high in terms of flow per gram weight of tissue, exceeded by only the brain and the kidneys. In fact, this study found splenic blood flow to be still higher than these reported values. Mean flow in the fully recovered rat was about 7 ml/min, and with observed transient increases to as high as 21 ml/min. The reason for these differences in reported values lies in the method by which blood flow was measured. Previous investigators have used radioactive microspheres. This method is dependent upon the microspheres becoming entrapped in the capillaries of the organ. In most cases this gives a very good estimate of blood flow. However in the spleen, there are fast arterio-venous pathways, and even evidence for arteriovenous shunts (Levesque & Groom, 1981). A proportion of the microspheres would thus pass right through the spleen. This would result in an artifactually low estimate of total organ blood flow.

At the time of surgical implantation of the flow probes, splenic arterial blood flow was not significantly different from splenic venous flow. However, as the animals recovered from surgery, the differential between splenic venous and arterial flow increased so that, at 10 days, only 70% of the fluid entering the splenic circulation, left in the venous effluent. Since this amounts to about 2.5 ml/min, and since this difference between inflow and outflow was observed to persist for at least one hour, it is concluded

that the extracted fluid cannot possibly be accommodated within the spleen i.e. *large volumes of lymphatic fluid must continuously drain from the spleen.* Lymphatic fluid from the splanchnic circulation generally flows through a series of ducts, ultimately reaching the thoracic duct. By contrast, much of the lymphatic fluid from the spleen, drains into a duct that taps into the hepatic portal vein (Job, 1915). Although this study found splenic lymphatic flow alone to be considerably higher than thoracic flow rates of 12.5 ml/hr previously reported in the rat (Kim & Bollman, 1954), it must be pointed out that no one has measured flow through the lymphoportal duct. It has been observed that, if the hepatic portal vein is partially occluded, lymphatic fluid is seen to back up into the splenic vascular arcade (unpublished observation). It is possible therefore that the spleen has an accessory lympho-venous cap to accommodate the very high rate of lymph flow from the organ.

It might be argued that such a large difference between arterial and venous flows indicates that vascular isolation of the spleen was incomplete. There are two possibilities: that some blood from the splenic artery might have supplied areas outside the splenic circulation, or some splenic venous blood did not flow into the splenic vein captured in the flow probe. Against this, it should be pointed out that, at the time of surgery, splenic arterial and venous flows were almost equal. It is extremely unlikely that, over the five days following surgery, vessels would develop *de novo* from the splenic artery to supply extrasplenic vascular beds. Nor was there any reason to suspect the formation of new pathways for venous drainage from the spleen, since splenic venous blood flow was

always robust. Moreover, examination of the splenic circulation at the time of sacrifice of the rat, never revealed any sign of extrasplenic blood vessels.

At one time it was believed that there were no deep lymphatics in the murine spleen (Maximow & Bloom, 1941; Yoffee & Courtice, 1970). It is true that they have been difficult to visualize. However, there is now ample evidence that they do exist (Pellas & Weiss, 1990). Moreover, it has been reported that there is strong lymph flow from the white pulp to deep lymphatic vessels in the periarterial lymphoid sheaths (Koshikawa, Asai & Iijima, 1984). Nor can one ignore our own observations that dye injected into the splenic artery appears rapidly in the splenic lymphatic ducts. The structural and functional morphology of the rat spleen is thus consistent with our observations that fluid is extracted from the splenic circulation into the lymphatic system.

In the previous paper showing that splenic venous hematocrit was higher than splenic arterial hematocrit, it was suggested that a larger difference might have been observed had the study been done on conscious animals (Kaufman & Deng, 1993). It was believed that high sympathetic tone at the time of surgery might have altered splenic circulation. The results reported in this paper confirm this suspicion. The small, but significant arterio/venous difference in hematocrit that had been observed in anesthetized animals could not be shown to be associated with a significant difference in blood flows, probably because of the greater variability inherent in measurement of blood flow compared with measurement of hematocrit. However, after recovery from surgery, the

differential in blood flow (lymphatic outflow) did increase significantly; one would presume that the arterio/venous differential of hematocrit also increased markedly during this period. The fact that high splenic lymphatic flow has not been previously reported might be attributed to the fact that these studies are the first experiments in which splenic afferent and efferent blood flows have been examined in conscious animals.

There are reports that pentobarbitone anesthesia decreases cardiac and renal sympathetic nerve activity in cats (Matsukawa, Ninomiya & Nishiura, 1993). The data from this study found that although anesthesia alone did reduce splenic blood flow, it did not significantly alter splenic extraction of fluid. It must therefore be the surgical procedure that is primarily responsible for perturbing the system. Several possibilities should be considered. Although the experiments have attempted to maintain fluid balance in the rats during surgery, it is conceivable that, at the end of the surgical procedure, they are somewhat dehydrated. There is evidence that hemorrhage decreases splenic blood flow and fluid efflux (unpublished observation). Surgery-induced hypovolemia could thus have caused low splenic blood flow and splenic fluid efflux that was observed on day 0. In addition, the splenic vessels are found to be extremely sensitive to being handled during surgery. It is possible that, in response to the flow probes being placed in position, the splenic vessels constricted, thus directly decreasing blood flow.

It was recognized long ago that surgical preparation of an animal for micropuncture raises the hematocrit by 6-10% (M dox, Price & Rector, 1977). There is

also evidence that plasma vasopressin levels, which are known to be elevated during surgery, are increased according to the extent of surgical invasiveness (Corman & Geelen, 1992). This latter study showed that, whereas induction of anesthesia had very little effect on vasopressin secretion, skin incision and insertion of femoral cannulae caused about a 4-fold increase in plasma vasopressin levels. More invasive surgery, namely tracheostomy, laparotomy and renal exposure, further increased circulating levels to 60 times the resting value. The same mechanism that underlies this response could also be responsible for mediating the reduction in splenic blood flow and extravasation that had been observed during implantation of the flow probes. In the case of the study on cats quoted above (Matsukawa, Ninomiya & Nishiura, 1993), it could be that the procedure of intubating and ventilating the animals after inducing anesthesia, initiated reflexes that depressed renal and cardiac sympathetic nerve activity.

Splenic blood flow and the arterio-venous difference fell in response to administration of sodium pentobarbitone. Since mean arterial blood pressure also fell in response to the anaesthetic, it should be considered as to whether changes in splenic circulation are secondary to changes in splenic perfusion pressure. However, the data reveal that, although blood pressure remained significantly depressed for the entire experimental period, splenic blood flow had returned towards control levels within 20 min, and splenic fluid extraction was not significantly altered. Moreover, although blood pressure was much lower in the pentobarbitone-injected animals (86 mm Hg) on day 5 than at the time of surgery (99 mm Hg), splenic blood flow and fluid extraction were

significantly higher. There was thus poor correlation between mean arterial pressure and splenic blood flow, and no correlation between mean arterial pressure and splenic fluid extraction.

However, it is concluded that there is no simple relationship between mean arterial pressure and splenic blood flow, the possibility cannot be ruled out that hemorrhage might induce changes in splenic perfusion pressure; given the extreme vasoactivity of the splenic vessels (unpublished observation), there could be reflex constriction of the splenic artery, so that splenic perfusion pressure at the point of entry of the splenic arteries into the substance of the spleen could well be reduced.

The previous experiments had shown that stimulation of the atrial volume receptors increases splenic venous hematocrit (Kaufman & Deng, 1993). There is also evidence that hemorrhage reduces splenic blood flow and abolishes the arterio-venous flow difference (unpublished observation). It is not unreasonable to propose that these changes are mediated by changes in splenic sympathetic nervous activity. Although there is some data suggesting that the baroreceptors cause only minimal changes in splenic nerve activity (Tobey & Weaver, 1987), those experiments were done on surgically prepared, anesthetized animals, conditions under which the present studies also observed only small changes in splenic function. Thus, it is suggested that splenic denervation in *conscious* animals would increase splenic blood flow and fluid efflux under resting conditions, or at least prevent their decreasing in response to hypovolemia or surgery.

In conclusion, this study has shown that splenic blood flow in fully recovered conscious rats is even higher than previously reported. Of particular significance is the observation that fully 30% of fluid volume flowing into the spleen is removed from the circulating blood. Such a role for the spleen as a portal from the intravascular to the extravascular space has not previously been proposed. It is conceivable that the high blood flow and marked separation of red blood cells from plasma that this experiment has found in the spleen are associated with this organ's immunological and blood cleansing functions. However it is tempting to speculate that the spleen could also play a role in controlling intravascular volume. Such a concept seems all the more likely in light of our previous findings that splenectomy abolishes the ANF-induced reduction in blood volume.

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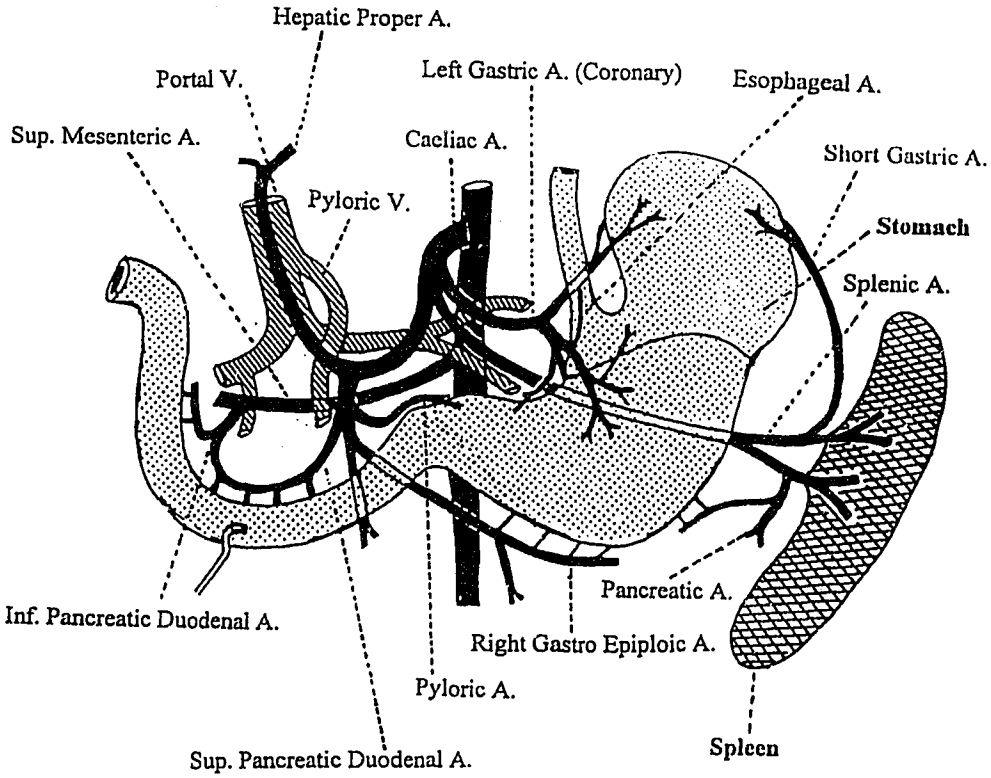

Figure 1. Anatomy of blood vessels in the vicinity of the spleen.

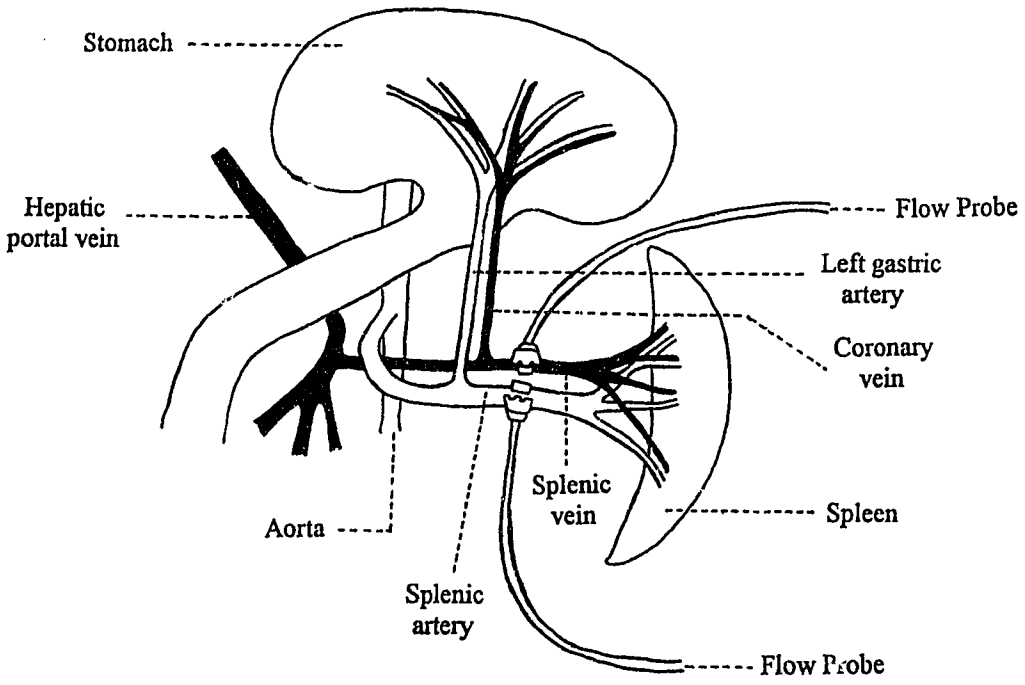

Figure 2. Placement of flow probes around splenic artery and vein.

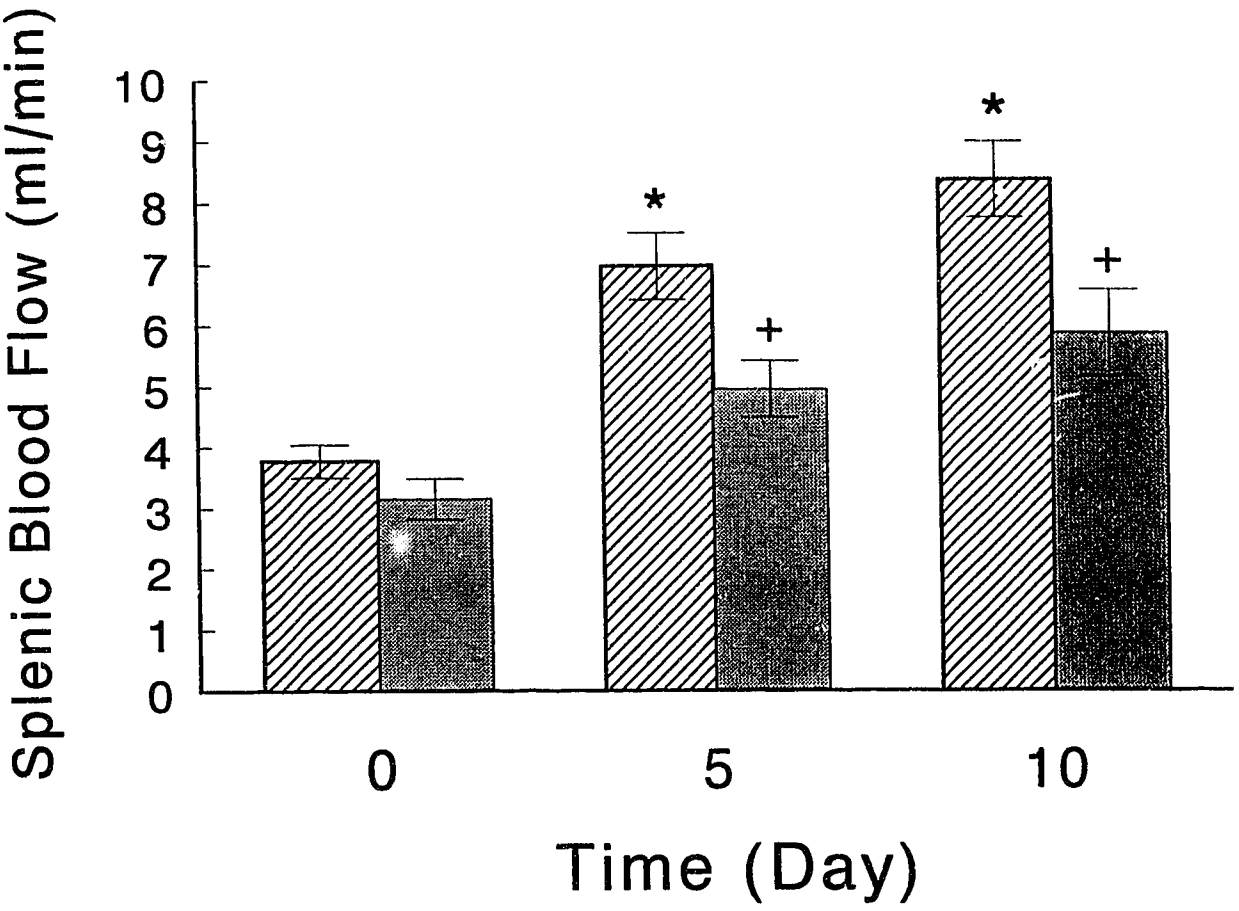

Figure 3. Mean splenic blood flows at time of surgery (0), and on days 5 and 10 of recovery. Hatch bar = splenic arterial blood flow. Solid bar = splenic venous blood flow. Significance of increase in blood flow relative to day 0 (surgery) was assessed by ANOVA and Student-Newman-Keuls test for multiple comparisons. *, +, relative to time 0, $P < 0.05$.

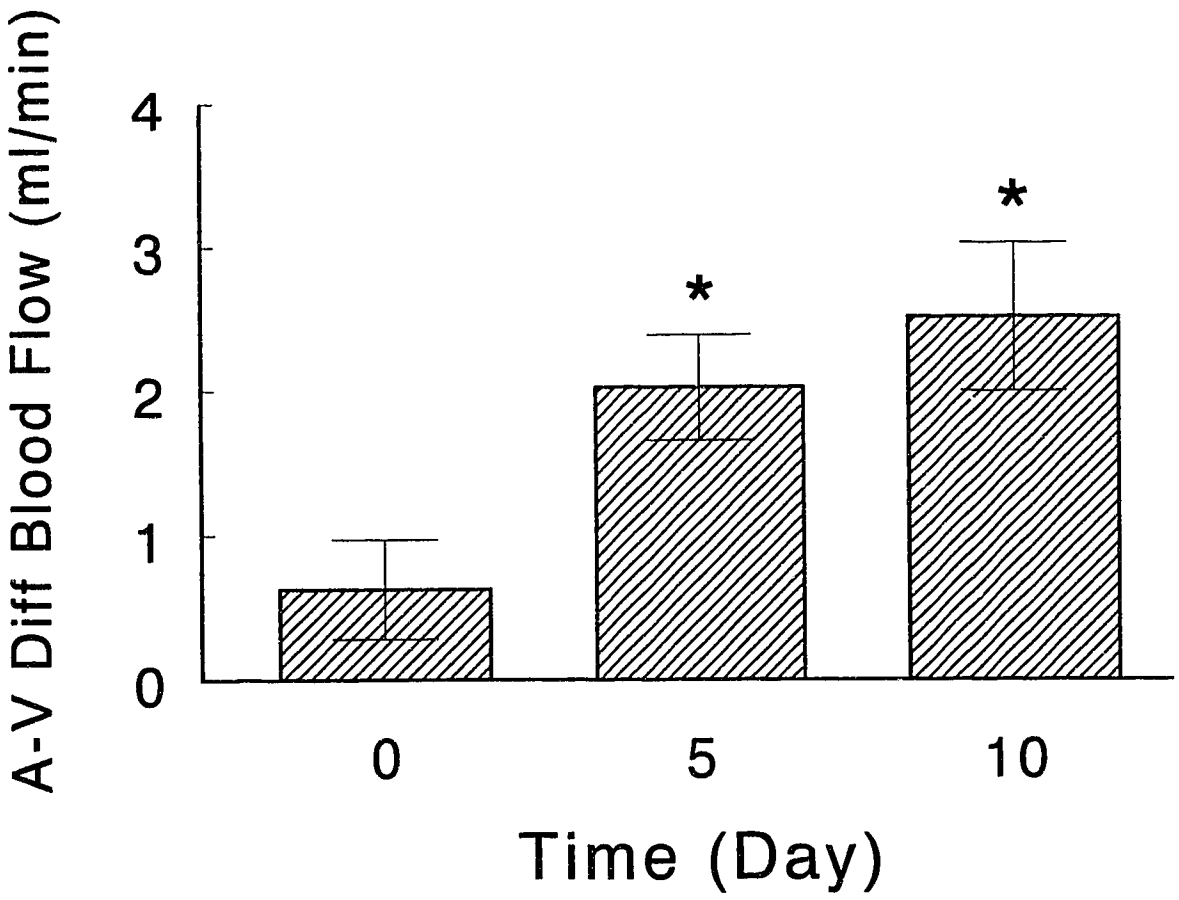

Figure 4. Difference between splenic arterial and venous blood flows (arterio-venous difference) on day of surgery (0), and on days 5 and 10 of recovery . The significance of the increase in arterio-venous difference was assessed using ANOVA, followed by Student-Newman-Keuls test for multiple comparisons. *, relative to time 0, $P< 0.05$.

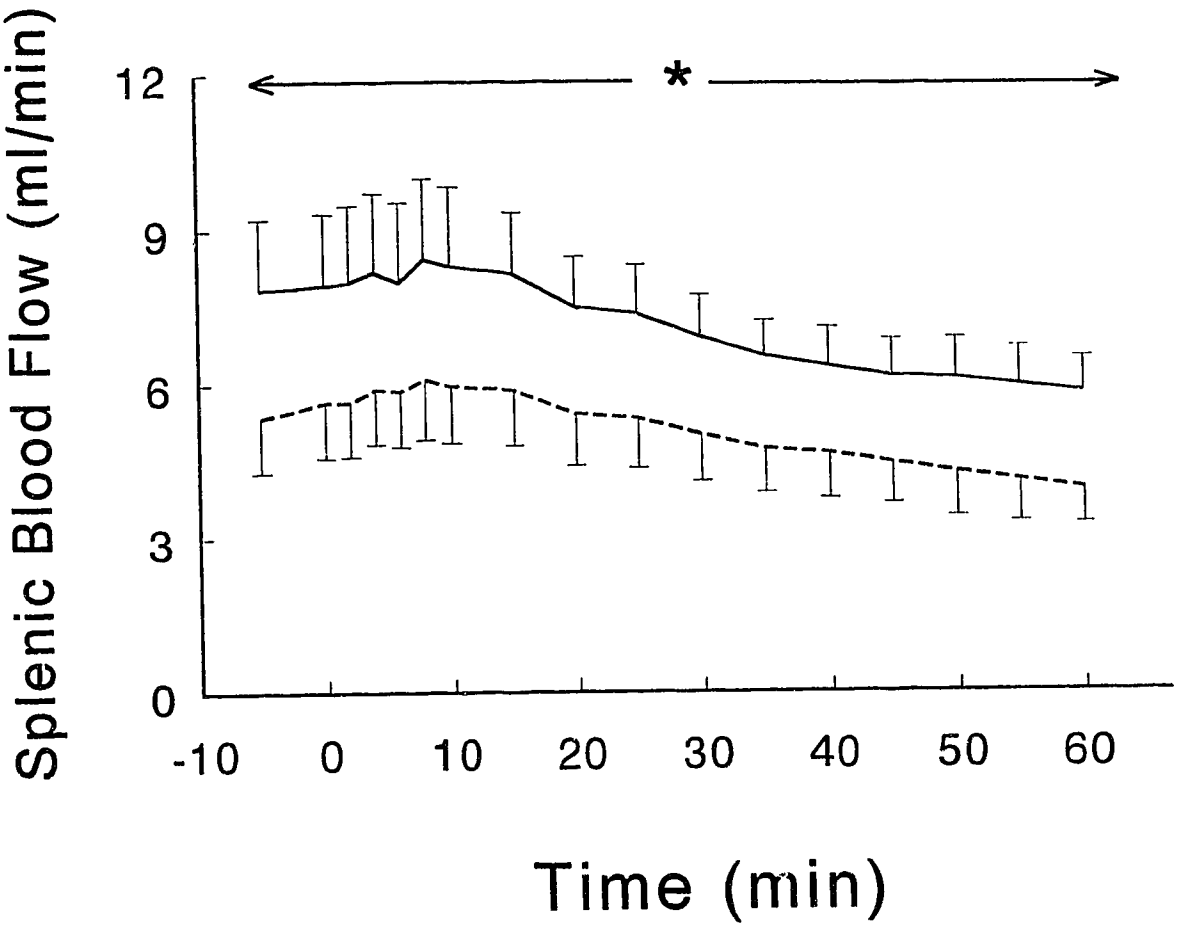

Figure 5. Splenic blood flow during the experimental period, recorded on day 5. Solid line = arterial flow. Broken line = venous flow. Significance of the difference between arterial and venous flows (arterio-venous difference) was assessed by Student's t-test for paired data and found to be significant throughout the experimental period. *, $P< 0.05$.

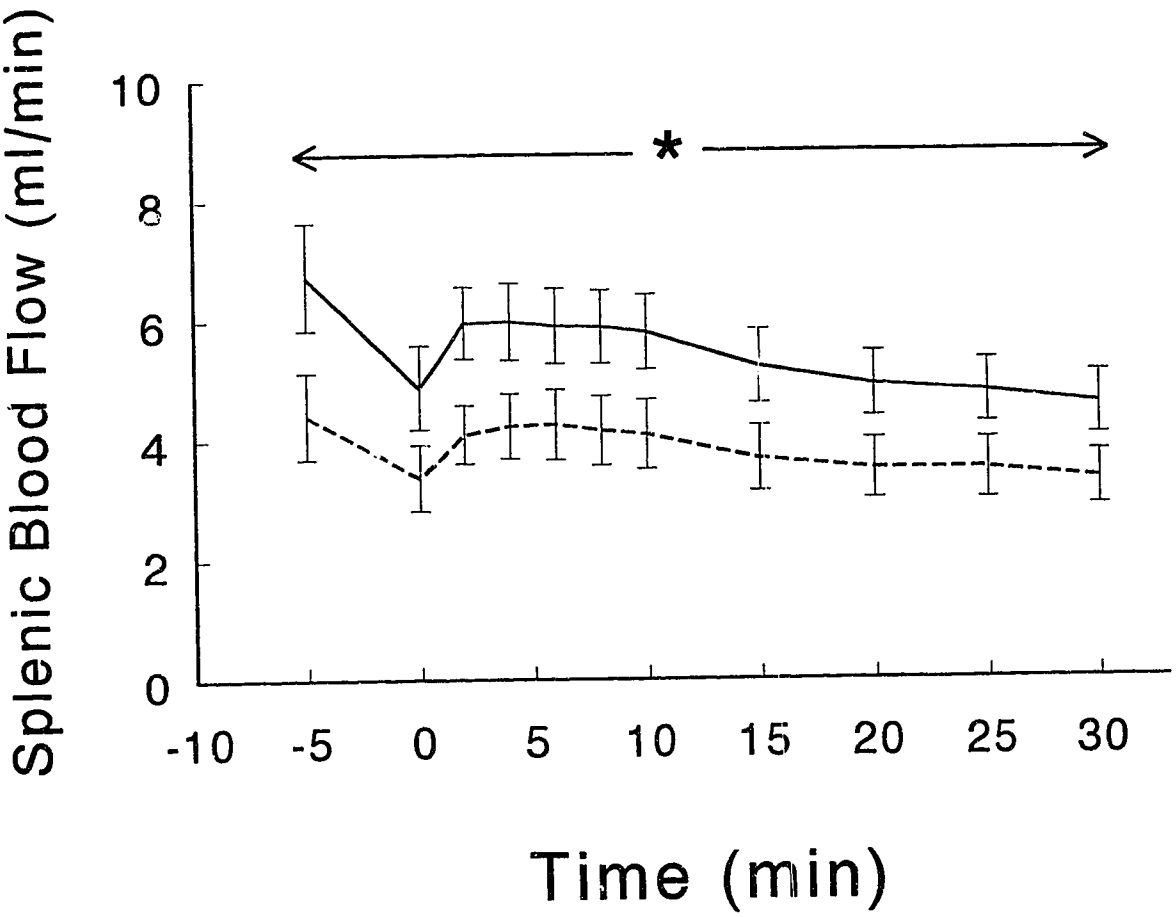

Figure 6. Effect of administering sodium pentobarbitone (20 mg, I.V.) on splenic blood flow. Arterial (solid line) and venous (broken line) blood flows are shown at the end of the 1 hr stabilization period (-5 min), immediately after infusing the anaesthetic (0 min), and for the remaining 30 min. of the experiment. Significance of the difference between arterial and venous flows (arterio-venous difference) was assessed by Student's t-test for paired data and found to be significant throughout the experimental period. *, $P< 0.05$.

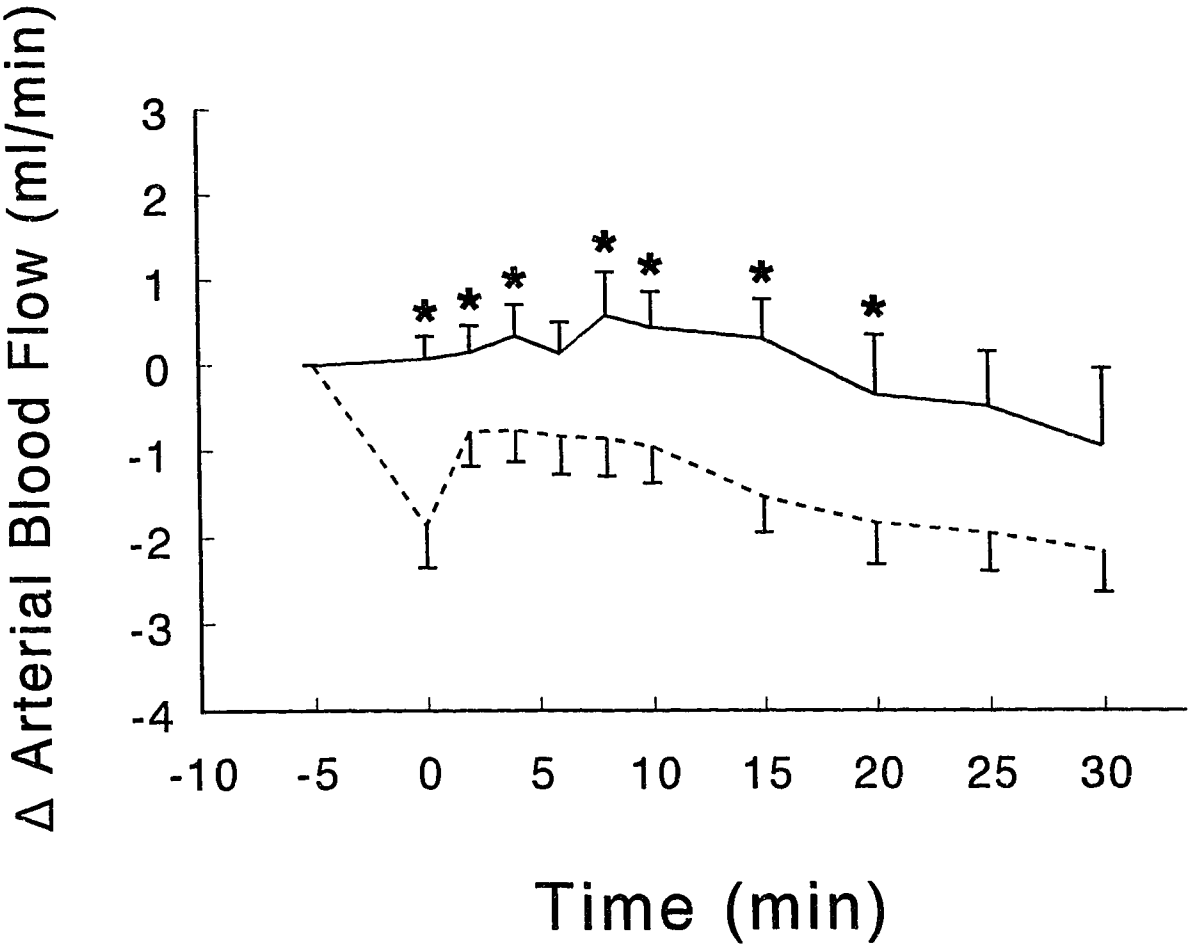

Figure 7. Effect of administering sodium pentobarbitone (20 mg, I.V.) on splenic arterial blood flow. Solid line = control group. Broken line = group administered sodium pentobarbitone. The significance of the fall in blood flow was assessed using Student's t-test for unpaired data. *, $P< 0.05$.

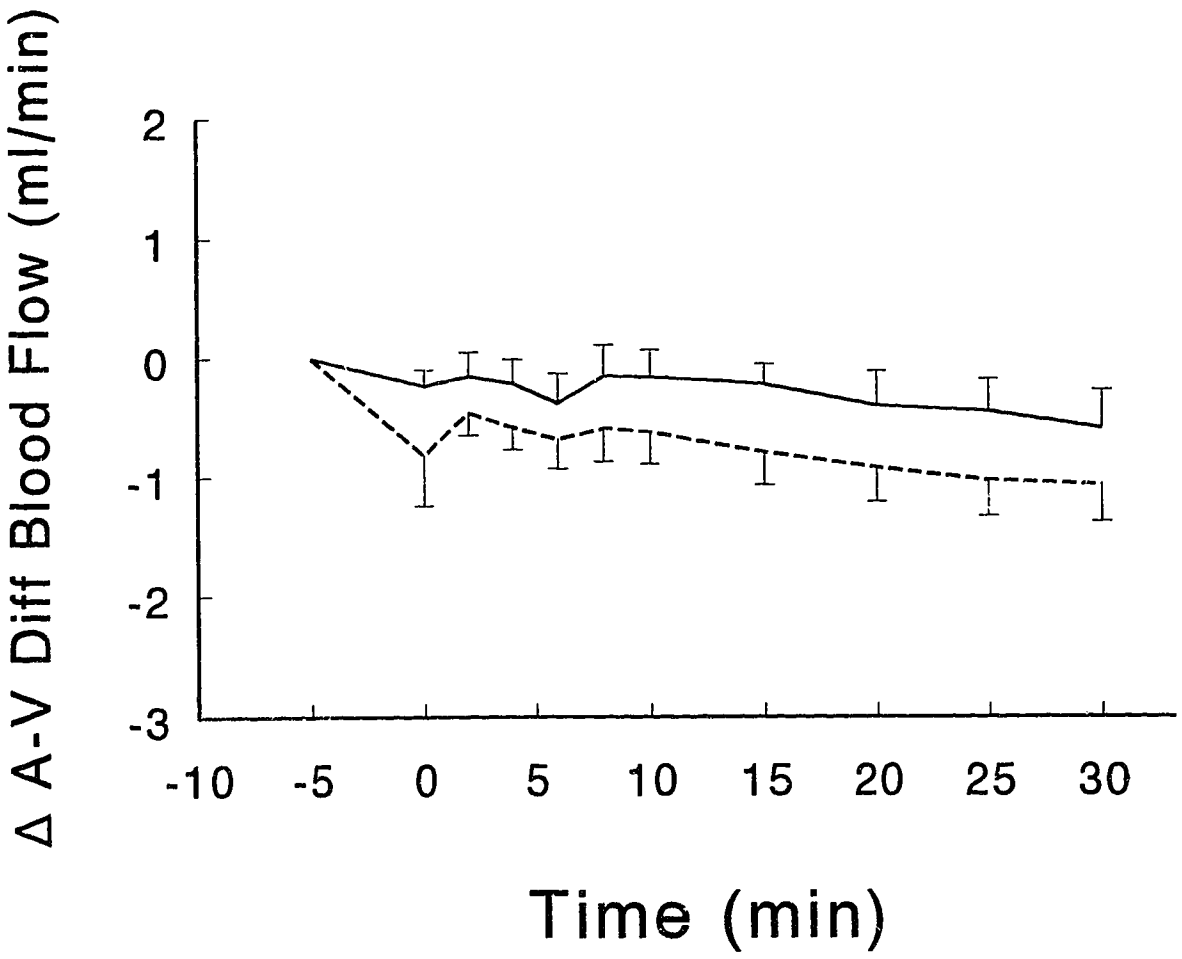

Figure 8. Effect of administering sodium pentobarbitone (20 mg, I.V.) on the difference between splenic arterial and venous blood flows (arterio-venous difference) on day 5 post surgery. Solid line = control group. Broken line = group administered sodium pentobarbitone. There is no significance difference between two groups.

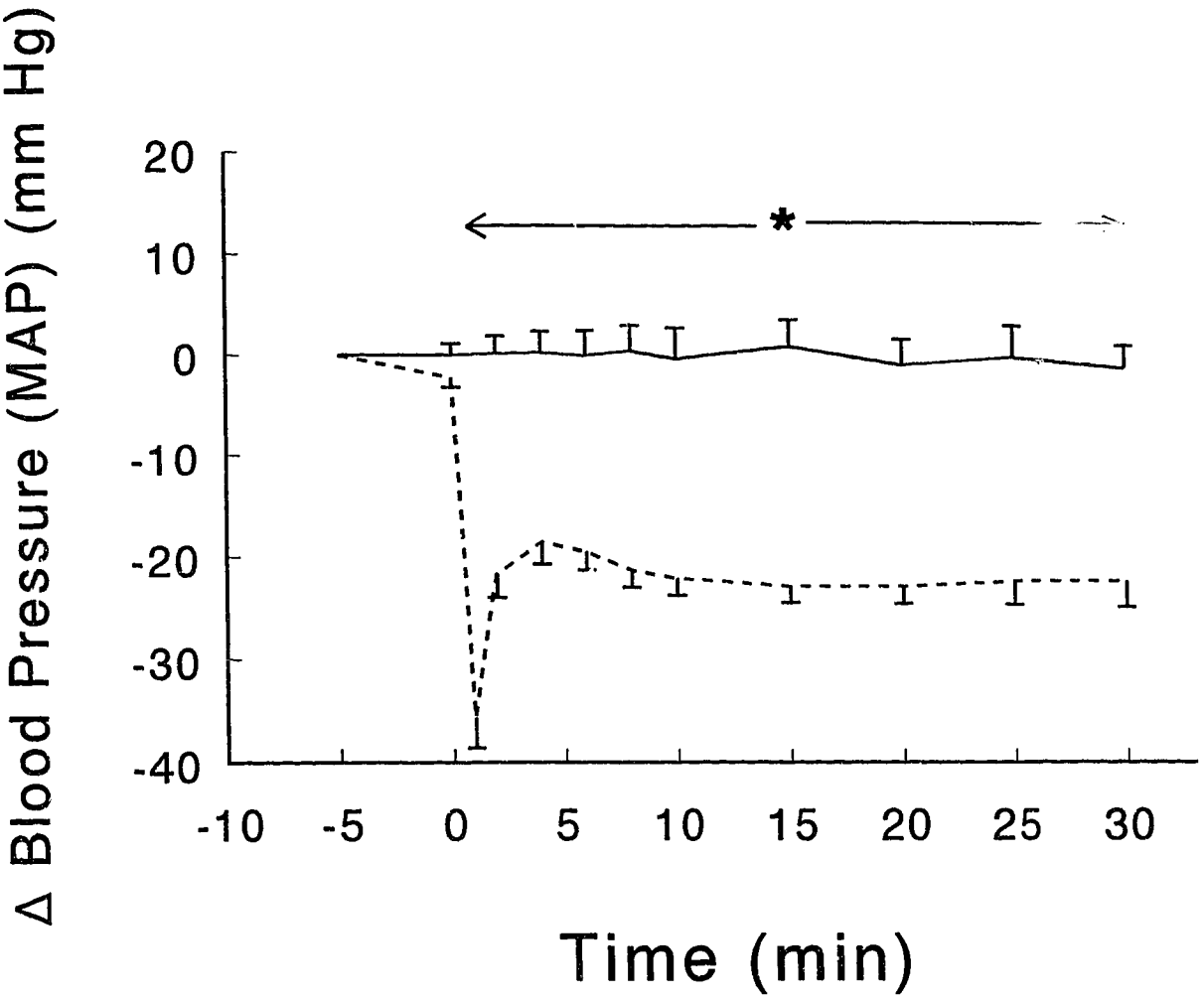

Figure 9. Effect of administering sodium pentobarbitone (20 mg, I.V.) on mean arterial blood pressure. Solid line = control group. Broken line = group administered sodium pentobarbitone. The significance of the fall in blood pressure was assessed using Student's t-test. *, $P< 0.05$.

CHAPTER 3

SPLENIC HEMODYNAMIC CHANGES DURING HEMORRHAGE

CHAPTER 3

SUMMARY

1. A direct study of the rapid changes in splenic circulation after volume-controlled hemorrhage was developed. The objective of this study was to investigate splenic hemodynamic and intrasplenic filtration function in fully recovered, awake and unrestrained rats with a shed blood volume of 1.5% body weight.

2. Rats were prepared with femoral artery and vein cannulae and blood flow probes (Transonic) around the splenic artery and vein. Ten days later, the conscious rats were bled (n=7) through the femoral venous cannula over a period of 3-4 minutes, while the control group (n=8) was tested as sham hemorrhage; mean arterial pressure (MAP) was maintained above 60 mmHg to ensure a survival rate of 100%. Splenic arterial and venous blood flow were measured simultaneously and directly, so that the difference of the splenic inflow and outflow blood volume would be computed throughout the hemorrhage during the ensuing 1 hour experimental period.

3. Before hemorrhage, splenic arterial blood flow was significantly higher than splenic venous blood flow (7.9 ± 1.1 ml/min vs 5.1 ± 0.8 ml/min). Immediately following hemorrhage, both splenic afferent and efferent blood flow fell, and the difference between splenic afferent and efferent flow was transiently abolished. Ten minutes after hemorrhage, absolute blood flows and the arterio-venous difference returned

towards pre-hemorrhage levels. MAP declined significantly from 102.3 ± 6.9 mmHg to 60.7 ± 7.4 mmHg when the blood was withdrawn, and then recovered slightly during the following hour.

4. These results suggest that, in response to hemorrhagic hypovolemia, there is not only hypoperfusion of the spleen, but also a decline in fluid efflux from the splenic circulation.

INTRODUCTION

It is well known that during hemorrhage, the principal changes are related to the cardiovascular system. Cardiac output and arterial blood pressure initially decrease as a result of blood loss. Through multiple neural and hormonal responses to hemorrhagic hypovolemia, such as reduction of vagal tone and enhancement of sympathetic tone, release of renin and angiotensin, increased aldosterone and ADH secretion, and increased heart rate and myocardial contractility, arterial blood pressure is restored towards normal (Schmidt & Thews, 1980).

Redistribution of blood flow between organs during hemorrhagic shock is thought to occur at the expense of the gastrointestinal organs, liver, spleen and kidneys, in order to maintain circulation to the brain and heart (Shoenberg et al, 1985; Blahitka & Rakusan, 1977). Although reports show that splenic venous hematocrit increases under hemorrhagic hypotension, splenic autotransfusion should not be counted as the only splenic response to hemorrhage. This is especially relevant in the case in human or murine hemorrhagic shock (Hoekstra, Dronen & Hedges, 1988) since, in both species, structural and functional studies show that there is extremely limited storage capacity for blood. However, as one of the most vascular organs in the body, the role of spleen and its hemodynamic changes during hemorrhage cannot be ignored. It is well known that the spleen is important for the maintenance of effective immunological defense mechanisms

to prevent complications induced by hypoperfusion (Cooper & Williamson, 1984). Increased release of platelets from the spleen to limit blood loss during hemorrhage is another important mechanism induced by the development of severe hypovolemia (Iversen, Benestad & Nicolaysen, 1992). Furthermore, previous results suggested that the spleen is one of the regulators that controls the extracellular fluid volume; under euvolemic conditions, there is intrasplenic filtration of red cell-free, isooncotic fluid out of the blood and into the lymphatic system (Chen & Kaufman, 1995). It was hypothesized that, in response to hemorrhagic hypovolemia, both splenic afferent and efferent blood flow would decrease and that the arterio-venous differential (fluid efflux) would fall.

The physiological response to hemorrhagic hypovolemia is very complicated, since it involves neural and hormonal responses. Not only does sympathoadrenal discharge increase, but also sympathetic activation is enhanced. There is a report showing that the early reflex responses to hemorrhage in regional sympathetic nerves is unidirectional sympathoexcitation, but that this is followed by a gradual return to the pre-bleeding level. These responses are of different magnitudes in different innervated organs, such that there was actually sympathoinhibition in the renal nerves (Leskinen et al, 1994; Koyama et al, 1992). The effects on splenic circulatory homeostasis have not been established.

A variety of reports have claimed that sympathetic nerves and catecholamines produce contraction or spasm of lymphatics *in situ* (Mawhinney & Roddie, 1973). It is well known that the lymphatic system represents an accessory route by which fluids can flow from the interstitial space into the blood. The most important role of all is that the lymphatics can carry proteins and large particulate matter away from the tissue spaces, neither of which can be removed by absorption directly into the blood capillary. The concentration of protein in the blood determines the body oncotic pressure which is one of the main factors determining the distribution of body fluid. Reports also indicate that there is strong lymph flow from the white pulp to deep lymphatic vessels in the periarterial lymphoid sheaths (Koshikawa, Asai & Iijima, 1984). Previous results showed not only that, when dye was injected into the splenic artery, it appears rapidly in the splenic lymphatic ducts, but also that red cell-free fluid is extracted from the splenic circulation into the lymphatic system (Kaufman & Deng, 1993; Chen & Kaufman, 1995). Therefore, it is reasonable to propose that the transvascular efflux of fluid from the splenic microvessels and the formation and flow of lymph would be reduced as a result of hemorrhage.

These experiments were designed to investigate the role of the spleen in cardiovascular performance under hemorrhage. Splenic circulation, rapid hemodynamic changes and intrasplenic filtration function during fixed volume hemorrhage could be directly determined in fully recovered, unrestrained and conscious rats. In this way, the complications commonly encountered in animal studies on the effects of hemorrhage,

such as the effect of anesthesia on the blood flow, high sympathetic tone, and high plasma vasopressin levels induced by invasive surgery, could be avoided. Furthermore, it was believed that this kind of simple reproducible model would allow study of the pathophysiologic processes during hemorrhage in humans.

MATERIALS AND METHODS

This experimental protocol was examined by the University of Alberta Animal Welfare Committee, and found to be in compliance with the guidelines issued by the Canadian Council on Animal Care.

ANIMALS:

Fifteen adult male Long Evans rats weighing between 225 and 250 grams were obtained from Charles River (St. Foy, Quebec). These rats were held in the University Animal Facility, in a temperature and humidity controlled room, with a 12:12 hour light:dark cycle. None of the surgical or experimental procedures were started until the rats weighed between 450 and 600 gram. The rats were maintained on a 0.28% sodium diet (Purina) and tap water *ad libitum*. All animals were fasted for 24 hours post-surgery to minimize digestive enzyme secretion, but they had free access to tap water. They were then held in the same conditions with an additional complete high-energy liquid nutrition food (Ensure Plus) during 10-day recovery period. Although not all the surgical animals

regained their pre-operative weights after 10 days recovery, no animal with more than 15% of body weight loss was used for this study. Since all the animals being used for this study were huge and there was a lot of fatty tissue stored in the abdomen cavity, 15% of the body weight loss did not represent a significant physiological stress.

DRUGS:

All surgical procedures were carried out under sodium pentobarbital anesthesia (60 mg/kg body weight, IP) and Atropine (0.12 mg/kg body weight, SC). A standard dose of Dihydrostreptomycin (Derapen-C, 0.22 ml, IM) was given before operation. In order to minimize problems resulting from damage to the pancreatic tissue during the surgery, a standard dose of Trasylol (0.3 ml, IV) was given. Also a few drops of Xylocaine (Lidocaine 10 mg/ml), which dilated the contracted vessels, was applied on the splenic vessels after implanting the flow probes. Following surgery, Buprenorphine (0.02 mg/kg body weight, SC) was administered every 8 hours for 2 days.

SURGERY:

Cannulation of femoral artery and vein: Two cannulae, Silastic (Dow, 0.51 mm ID, 0.94 mm OD) and Micro-Renethane (Braintree, 0.30 mm ID, 0.64 mm OD) were implanted respectively in the femoral vein and artery. The femoral arterial cannula was used for monitoring the changes of mean arterial blood pressure during hemorrhage. The femoral venous cannula was used for withdrawing blood. Both cannulae were

exteriorized at the nape of the neck via stainless steel tubing, and filled with approximately 200 µl of heparinized saline (10,000 units/l) to maintain patency.

Implantation of blood flow probes: A midline laparotomy was performed, extending up to the xiphisternum from the lower part of the abdomen. The stomach was pulled out and wrapped in a saline soaked 4 x 4 inch gauze, reflected up and laid on the thorax of the rat. The spleen was carefully cleared from its attachments by dividing the ligaments extending between spleen-diaphragm and spleen-stomach. The vessels in the gastrosplenic ligament i.e short gastric vessels, and the vessels to the body and tail of the pancreas from the spleen were ligated and divided. Thus, the primary splenic artery and vein supplying and draining the splenic pedicle were the only remaining vessels to and from the spleen. Great care was taken in clearing the pancreatic and fatty tissue from the splenic artery and vein, in order to ensure proper acoustical coupling with the flow probes. The two flow probes (Transonic transit time ultrasonic 1 mm R-series probes, 1R), were glued together on a Silastic sheet, and placed under the splenic vascular arcade. The splenic artery and vein were slipped into individual probe windows. The stomach was then replaced into its original position over the splenic vessels and the flow probes. The connectors of the probes were led subcutaneously to the back of the neck.

EXPERIMENTAL PROTOCOL:

Nine days post-surgery, the day before each experiment, the rats were placed in metabolism cages with food and water *ad libitum*. (This facilitated access to the cannulae and probe leads). On the following day, the femoral arterial cannula was connected to the blood pressure transducer so that the changes of mean arterial blood pressure could be measured. The femoral venous cannula was connected to a syringe for withdrawal of blood. The blood flow probes were connected to an ultrasonic, transit time blood flowmeter (Transonic, N.Y.). All the rats were maintained unrestrained in their metabolism cages throughout the experimental period. Splenic afferent and efferent blood flow, and mean arterial blood pressure were recorded simultaneously by the Transonic flowmeter.

During the test period, the rats were kept in a quiet room, and observed through a window. After one hour of stabilization, basal measurement of splenic arterial and venous blood flows, and mean arterial blood pressure were recorded for 10 minutes. Then the rats were randomly divided into two groups: (a) hemorrhage group (n=7) - the rats were bled 1.5% body weight within 3-4 minutes, (b) control group (n=8) - the rats were subjected to sham hemorrhage, i.e. the syringe connected to the femoral vein cannula was picked up and held for the same duration as the hemorrhage procedures. Different animals were used for each group i.e. the animals did not act as their own controls. Splenic afferent and efferent blood flow, and mean arterial blood pressure were subsequently recorded every 2

minutes for 10 minutes and then at every 5 minute intervals for another 60 minutes. Splenic blood flow was reported as milliliters per minute.

STATISTICAL ANALYSIS:

The statistical significance of the difference between splenic afferent and efferent blood flow was calculated by Student's t-test for paired data. The significance of the changes in splenic blood flow and arteriovenous differential after hemorrhage were assessed by ANOVA, followed by Student-Newman-Keuls test for repeated multiple comparisons. The change of mean arterial blood pressure caused by hemorrhage was tested using Student's t-test for unpaired data. The level of statistical significance was defined at $P<0.05$. Data was expressed at means ± SEM.

RESULTS

After the one-hour stabilization period, mean splenic arterial blood flow was 8.9 ± 0.8 ml/min and mean splenic venous blood flow was 6.7 ± 1.0 ml/min (n=8) on day 10 post-surgery (Figure 1). Flow tended to fall slowly during the period of observation. However, when 1.5% body weight of blood was withdrawn, both mean splenic arterial and venous blood flow decreased dramatically and simultaneously. Splenic arterial blood flow decreased to 1.9 ± 0.5 ml/min (Figures 2 and 4), and venous blood flow decreased to

than splenic venous blood flow, the arterio-venous difference was significantly reduced from 2.8 ml/min to 0.9 ml/min (Figure 3). For at least 8 min following blood withdrawal, there was a significant decrease in the splenic arterio-venous difference in the hemorrhage group compared with the control group (Figure 6).

Following hemorrhage, both splenic arterial and venous blood flow tended to recover to pre-hemorrhage level. At 10 minutes after bleeding, splenic arterial blood flow recovered to about 4.6 ml/min which was approximately half of pre-hemorrhage arterial levels and remained stable thereafter (Figure 2); at 30 minutes, there was no significant difference between the control and hemorrhage groups (Figure 4). Splenic venous blood flow also recovered to about 2.9 ml/min during the same period (Figure 2).

In response to 1.5% body weight of hemorrhage, mean arterial blood pressure decreased significantly from 102 ± 6 mmHg to 61 ± 7 mmHg. Mean arterial blood pressure gradually recovered, and reached 73 ± 7 mmHg by the end of 1 hr experimental period. It was, however, still significantly depressed when compared to either the pre-hemorrhage mean arterial blood pressure level or the control group (Figure 7).

DISCUSSION

The physiological and/or pathophysiological role of the spleen during hemorrhage has not well been studied. Whether the splenic deep lymphatic system exists has been controversial. One reason for this confusion was due to the varied animal models used in studying spleen function. The spleen acts as blood reservoir in many species. Under splenic contraction, large quantities of blood are expelled into the circulation. However, the spleen in the human and rat have very little such function as a blood reservoir. In both species, capsular contractio. is limited due to the nonmuscular capsule of the spleen (Reilly, 1985). The rat is an ideal model in which to study splenic hemodynamic function because rats have the same elaborate sinusoidal network and fast blood flow pathways as humans, and RBC washout tests show that it has qualitatively similar patterns of blood flow as in humans (Cilento et al, 1980; Stock et al, 1983).

On day 10 post-surgery, blood flow to the spleen in fully recovered conscious unrestrained rats was found to be extremely high, expressed as flow per gram weight of tissue. Mean splenic arterial blood flow ranged from 7.9 ± 1.1 ml/min to 8.9 ± 0.8 ml/min (n=15, control group plus hemorrhage group). Mean splenic venous blood flow ranged between 5.1 ± 0.8 ml/min to 6.7 ± 1.0 ml/min (n=15, control group plus hemorrhage group). As the largest lymphatic organ, and indeed the only one specialized for the blood filtration and maintenance of immunological defense mechanisms (Cooper &

Williamson, 1984; Groom & Schmidt, 1990), this huge amount of blood flow per minute to the spleen was not unexpected. However, as discussed in the previous study (Chen & Kaufman, 1995), these values are far higher than have previously been reported.

Decreased cardiac output and total organ perfusion blood flow at the microcirculatory level are the initial cardiovascular responses to hemorrhage. Thus, when 1.5% of body weight of blood was withdrawn, both splenic arterial and venous blood flow markedly decreased. Mean splenic arterial blood flow dropped sharply from 7.9 ± 1.0 ml/min to 1.9 ± 0.5 ml/min while mean splenic venous blood flow dropped from 5.1 ± 0.8 ml/min to 0.9 ± 0.4 ml/min simultaneously (Figure 2). The significant and stable splenic arterio-venous blood flow difference decreased following hemorrhage from 2.8 ml/min to 0.9 ml/min (Figure 3). The decrease in splenic blood flow was presumably due to a reduction in splenic perfusion pressure. The decrease of splenic arterio-venous difference must reflect a decrease in intrasplenic filtration of intravascular fluid out of blood into lymphatic ducts. This response to hypovolemia would minimize intrasplenic filtration into the lymphatic system, thus protecting intravascular volume.

There is no doubt that splenic deep lymphatics exist (Barcroft & Florey, 1928; Han, van Krieken & te Velde, 1992; Pellas & Weiss, 1990) and that there is high lymph flow to these deep lymphatic vessels (Koshikawa, Asai & Iijima, 1984). Under physiological conditions, this splenic lymphatic fluid flows back to the vascular system

through the thoracic duct to the right heart, and through the lymphoportal duct to the hepatic portal vein (Job, 1915). Under hypovolemic hemorrhage, mesenteric lymphatic ducts in the rat are reported to act like blood vessels, i.e. circulating epinephrine and norepinephrine induce lymphatic constriction (Dabney, Buehn & Dobbins, 1988). This immediate translocation of protein-rich lymph back to the intravascular system, is important in maintaining plasma oncotic pressure, and hence blood volume. It is for this reason that some previous reports have concluded that the lymphatic circulation provides an important self-resuscitation mechanism which is called upon during hemorrhagic hypotension (Dabney, Buehn & Dobbins, 1988; Zhang, 1991).

After a transient decrease in splenic blood flow, both splenic arterial and venous blood flow recovered *towards* pre-hemorrhage level gradually within 10 minute after bleeding. However, the decrease of total blood volume in the body only allowed the splenic arterial blood flow to recover to 4.6 ± 0.6 ml/min and splenic venous blood flow to reach 2.9 ± 0.8 ml/min 10 minute after hemorrhage without any fluid resuscitation (Figure 2). The recovered splenic blood flows then remained stable throughout the experimental period. The splenic arterio-venous blood flow difference also recovered gradually to 2.4 ± 0.8 ml/min after 30 minutes post bleeding (Figure 3). It has been shown that the unidirectional sympathoexcitation and active peripheral vasoconstriction of the capacitance vessels in response to the early stage of hemorrhage are mediated by summate unloading of arterial baroreceptors and cardiopulmonary receptors. However, this unidirectional sympathoexcitation is later opposed by reflex sympathoinhibition,

probably mediated by the release of neurotransmitters such as opiate peptides from vagal afferent fibers (Koyama et al, 1992). It is believed that this reflex sympathoinhibition is one factor preventing further decline of splenic blood flow under hemorrhagic hypovolemia and maintaining spleen blood flow throughout the experimental period. This prevention of the progressive decline in splenic blood flow is similar to the reports of renal sympathoinhibitory effects which act to maintain renal function. In the case of the spleen, it is important to prevent impaired splenic function, since this organ is so important in maintenance of effective immunological defense mechanisms (Cooper & Williamson, 1984).

The sustained lymphatic contraction induced by the increasing levels of circulating norepinephrine and epinephrine would thus have increased lymphatic pressure during hemorrhage (Dabney, Buehn & Dobbins, 1988). Such an increase in lymphatic back pressure decreases the further formation of lymph (Drake & Gabel, 1991). In this hemorrhage model, no attempt was made to maintain the hypotension at certain level. During the hemorrhagic experiment, MAP decreased sharply from 102 ± 7 mmHg to 61 ± 7 mmHg during bleeding and then returned to 73 ± 7 mmHg (Figure 7). Therefore, only a transient decrease of splenic arterio-venous difference in response to the hemorrhage was observed. Had the period of hypotension at 60 mmHg been prolonged, one might have observed a stable decrease in the splenic arterio-venous differential blood flow. The recovery of splenic blood flow, despite the continued fall in mean arterial pressure, suggests the existence of an autoregulatory mechanism in the spleen similar to that found

in the kidney. It is very possible that the spleen has such a mechanism to maintain its intrasplenic filtration and immunological functions. It is significant that, as in the kidney, splenic blood flow was maintained only above a MAP of 60 mmHg (Vander, 1991).

Progressive hemorrhage-induced changes, such as tissue ischemia, cellular dysfunction, endotoxin infection, and immunological and metabolic dysfunction, are still major causes of morbidity and mortality. The increases in cardiac function and sympathetic tone, to maintain cardiac output and mean arterial blood pressure, are not the only compensatory responses to hemorrhage. Decreased urinary output, increased Na+ and water absorption, and increased fluid intake to replace the lost blood volume are also basic and effective ways to prevent the body falling into hemorrhagic shock. In this study, another aspect of self-resuscitation in the early stages of oligemia has been investigated, namely redistribution of the blood flow in a splanchnic organ, the spleen. The evidence from this study points to the lymphatic system, particularly that associated with the spleen, playing an important role in the homeostatic responses to hemorrhage.

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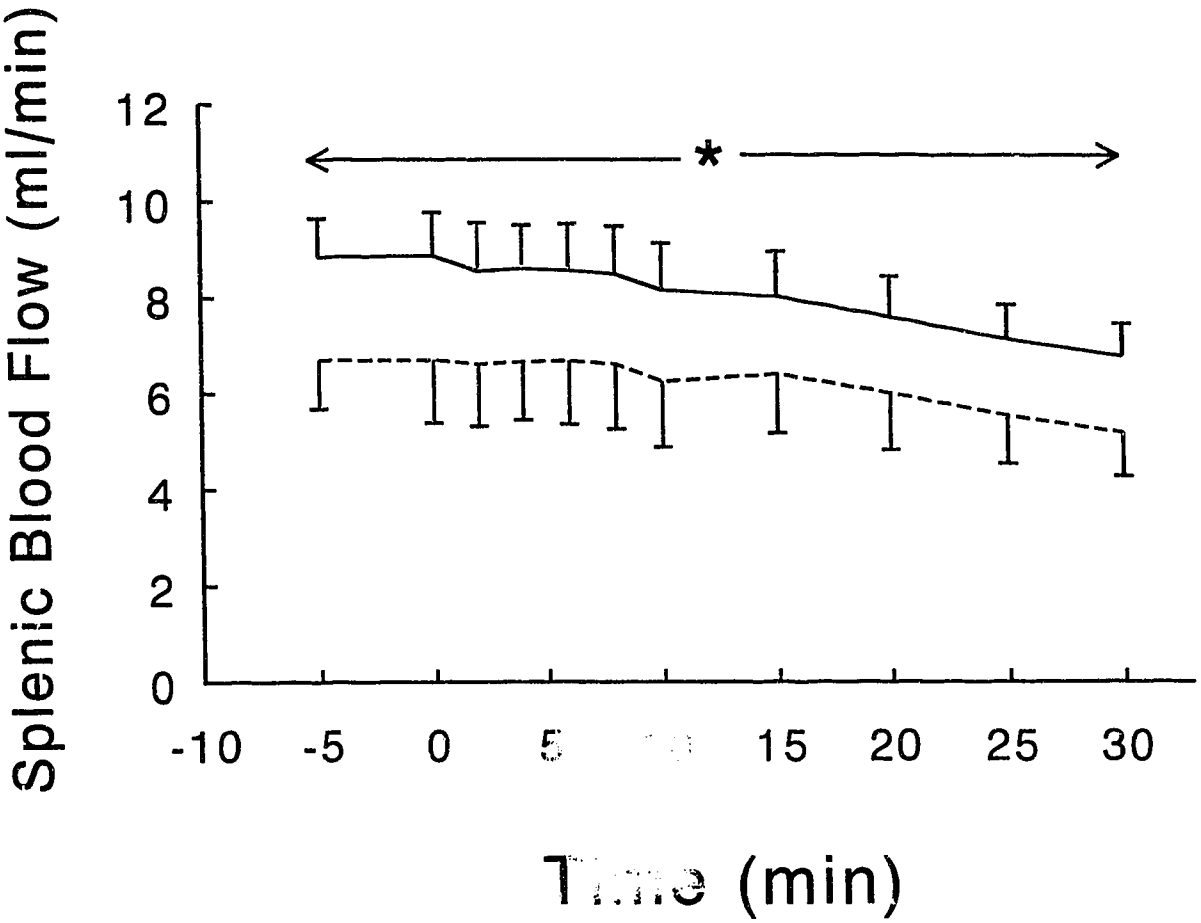

Figure 1. Splenic blood flow during the experimental period, recorded on day 10. Solid line = arterial flow. Broken line = venous flow. Significance of the difference between arterial and venous flows (arterio-venous difference) was assessed by Student's t-test for paired data and found to be significant throughout the experimental period. *, $P<0.05$.

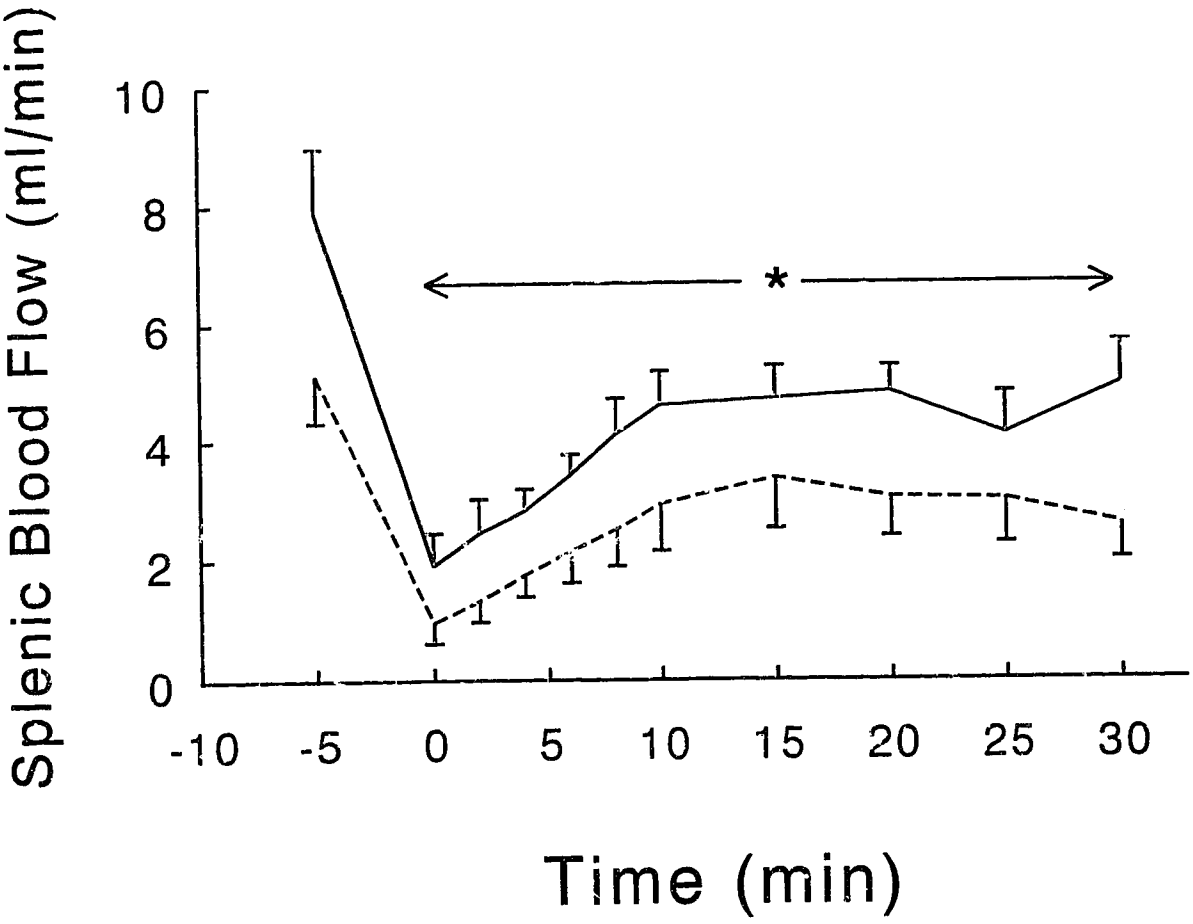

Figure 2. Effect of hemorrhage (1.5% BW, I.V.) on splenic blood flow. Solid line = arterial flow. Broker line = venous flow. Both blood flows are shown at the end of the 1 hr stabilization period (-5 min), immediately after bleeding 1.5% BW of blood (0 min), and for the remaining 30 min. of the experiment. Significance of the difference of blood flows comparing to the pre-hemorrhage values was assessed by ANOVA and Student-Newman-Keuls test for repeated multiple comparisons. It was found to be significant throughout the experimental period. *, $P<0.05$.

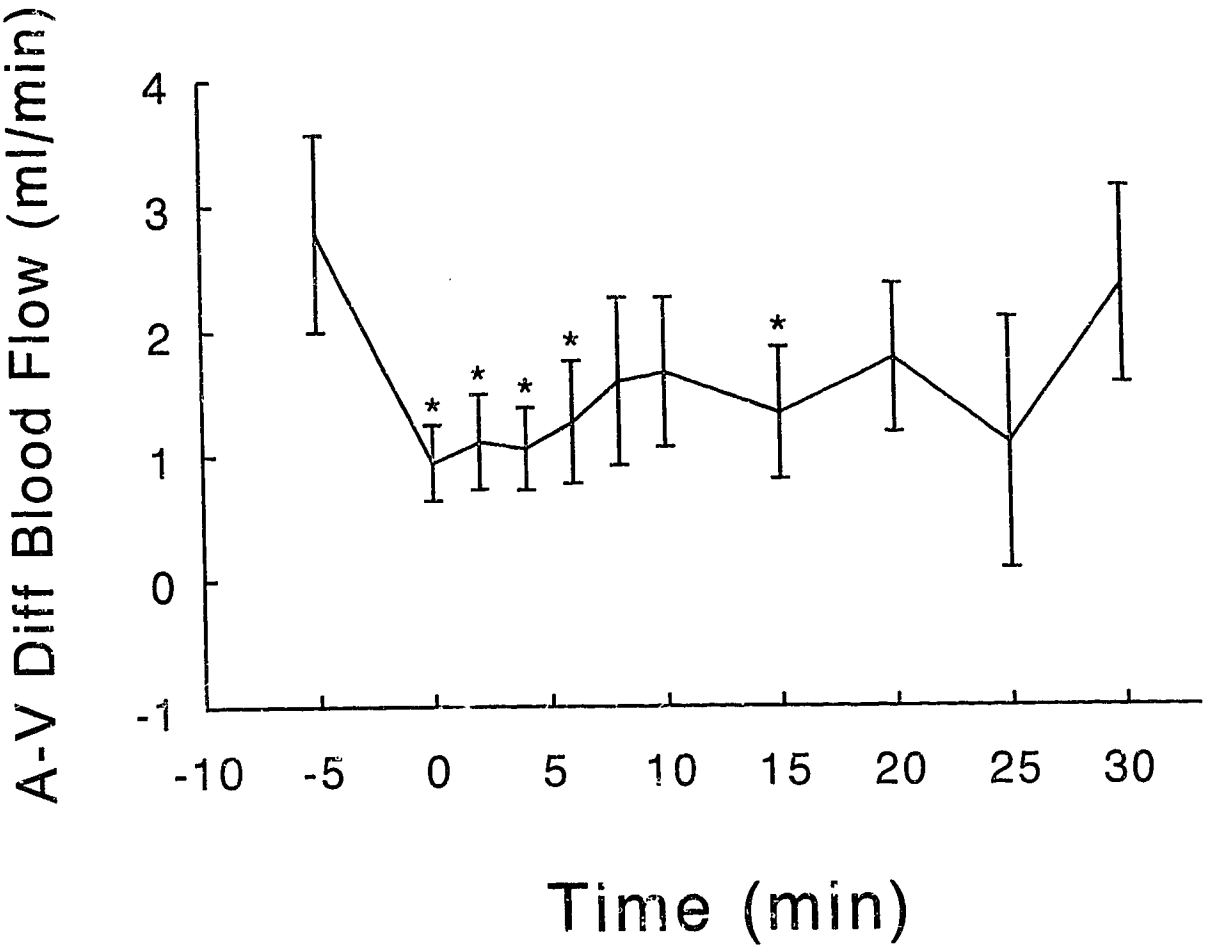

Figure 3. Effect of hemorrhage (1.5% BW, I.V.) on arterio-venous difference of splenic blood flow. Difference between splenic arterial and venous blood flow (arterio-venous difference) on day 10 of recovery is shown at the end of the 1 hr stabilization period (-5 min), immediately after bleeding (0 min), and for the remaining 30 min of the experiment. Significance of the arterio-venous difference comparing to the pre-hemorrhage value was assessed by ANOVA and Student-Newman-Keuls test for repeated multiple comparisons. *, $P<0.05$.

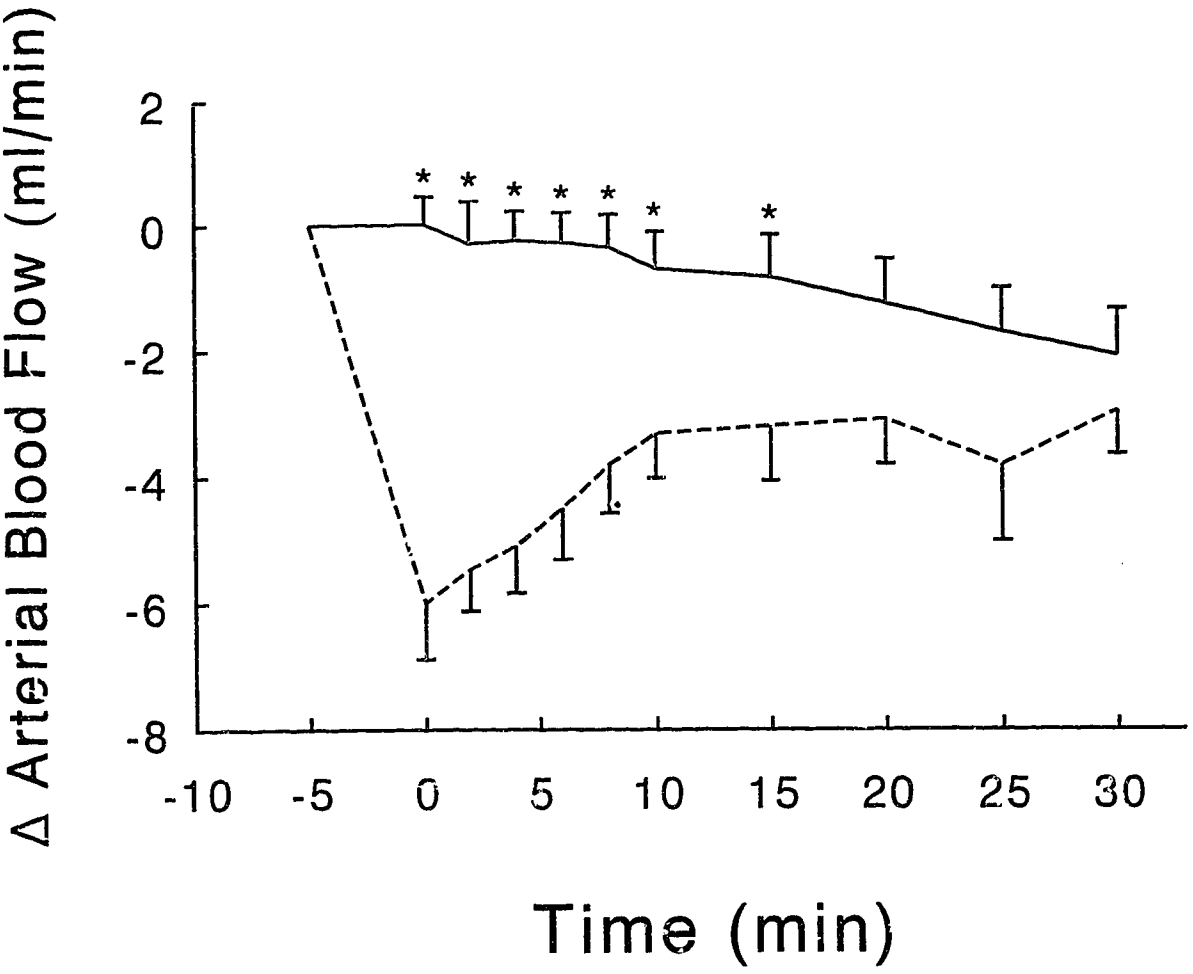

Figure 4. Effect of hemorrhage (1.5% BW, I.V.) on splenic arterial blood flow. Solid line = control group. Broken line = hemorrhage group. The significance of the fall in blood flow was assessed using Student's t-test for unpaired data. *, $P<0.05$.

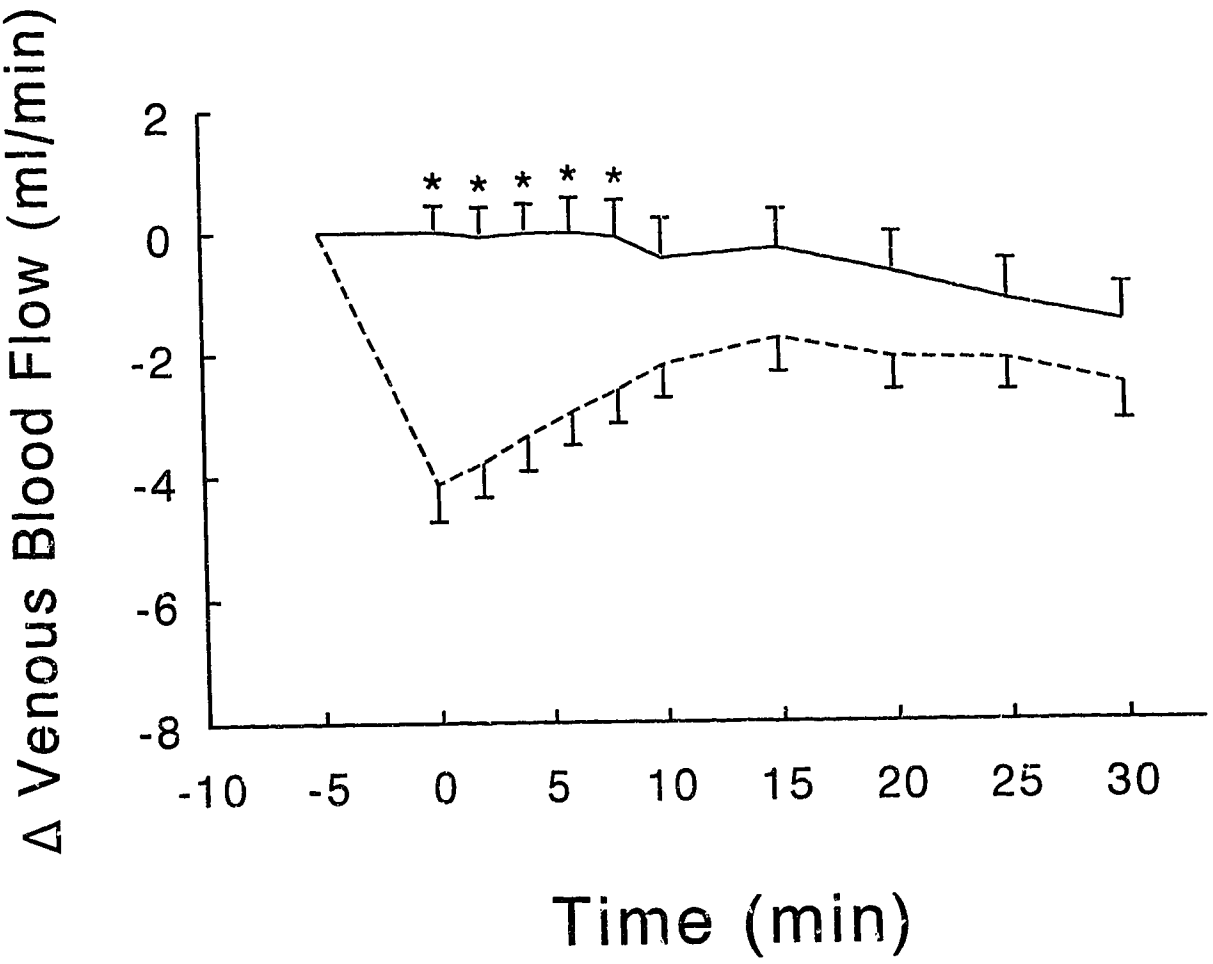

Figure 5. Effect of hemorrhage (1.5% BW, I.V.) on splenic venous blood flow. Solid line = control group. Broken line = hemorrhage group. The significance of the fall in blood flow was assessed using Student's t-test for unpaired data. *, $P<0.05$.

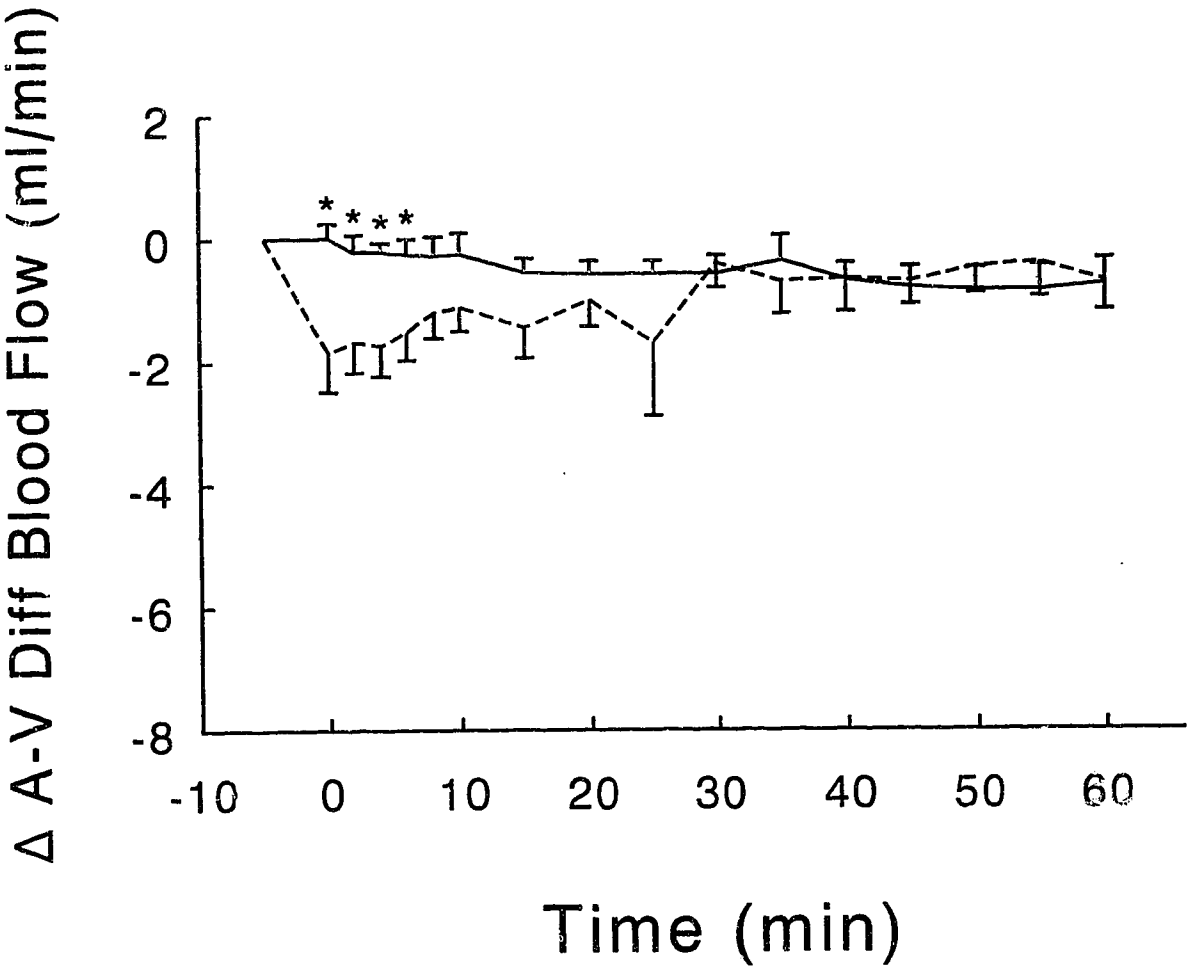

Figure 6. Difference between splenic arterial and venous blood flow (arterio-venous difference) on day 10 of recovery . Solid line = control group. Broken line = hemorrhage group. The significance of the fall in arterio-venous difference blood flow in hemorrhage group was assessed using Student's t-test for unpaired data. *, $P<0.05$.

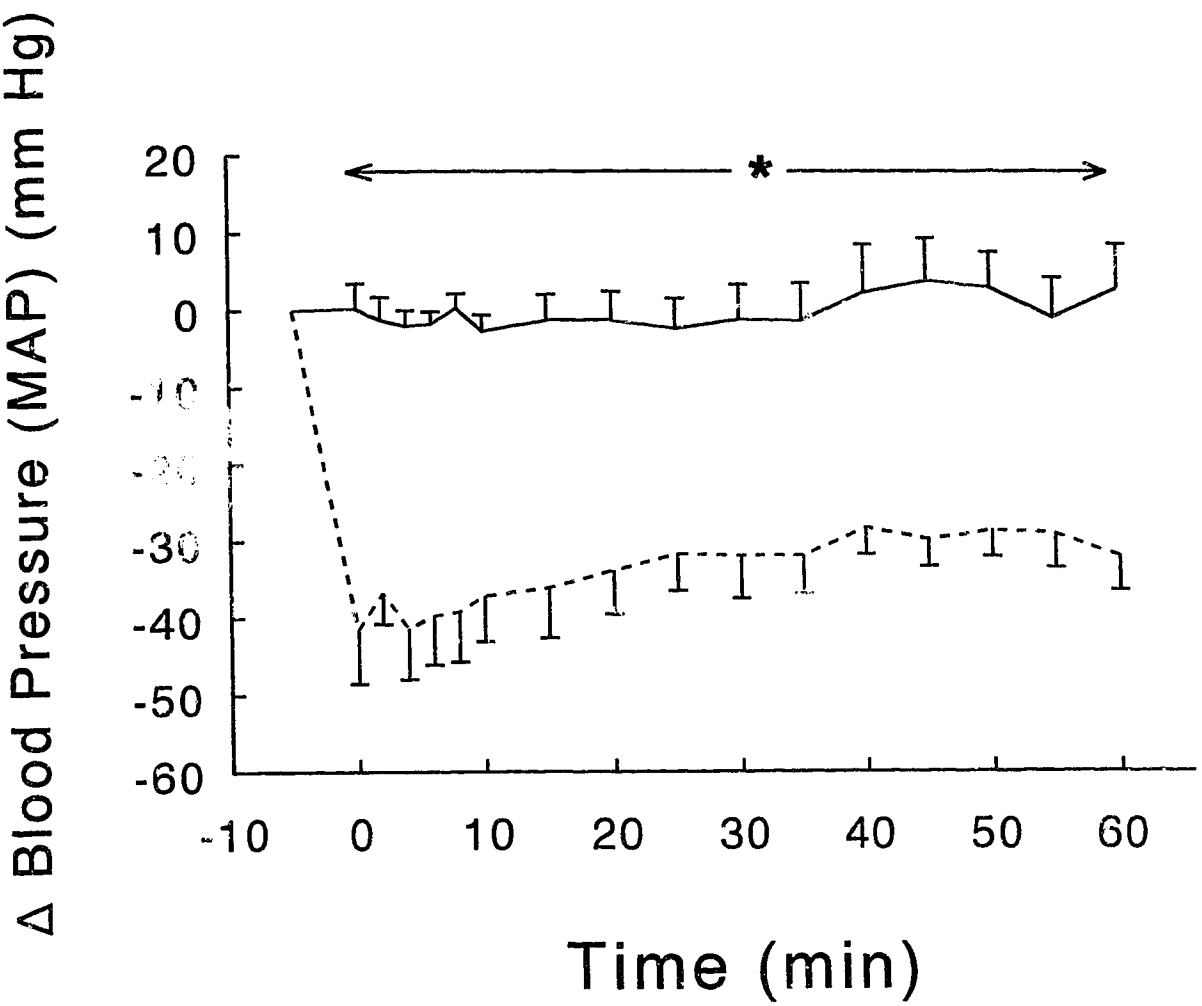

Figure 7. Effect of hemorrhage (1.5% BW, I.V.) on mean arterial blood pressure. Solid line = control group. Broken line = hemorrhage group. The significance of the fall in blood pressure was assessed using Student's t-test for unpaired data and found to be significant throughout the experimental period. *, $P<0.05$.

CHAPTER 4

GENERAL DISCUSSION

AND

CONCLUSION OF THE THESIS

CHAPTER 4

DISCUSSION AND CONCLUSION

The spleen, one of the most important vascular organs in the body, is known to be specialized for the filtration of blood (Groom & Schmidt, 1990). Although splenic function in immunological defence is well established, the traditional concept that the spleen's contribution to *cardiovascular* homeostasis and its role as a blood reservoir is now doubtful. One reason for this confusion about splenic structure and function lies with the various animal models that have been used. The spleen is indeed a blood reservoir in many species. Under contraction, large quantities of blood may be expelled into the circulation. However, the spleen in the human and the rat have very little such storage capacity. In these species, capsular contractile ability is very limited due to the nonmuscular capsule of the spleen (Reilly, 1985). Therefore, because measurements of splenic blood flow are not confounded by expulsion of the RBC, the rat is an ideal animal model in which to study splenic hemodynamic function. Furthermore, rats have the same elaborate sinusoidal network and fast blood flow pathways as humans, and the RBC washout test shows it to be qualitatively similar to humans (Groom & Schmidt, 1990).

Studies of splenic function have also been hampered by the nature of this organ. The spleen is a very fragile organ which is extremely vulnerable to autolysis (Han, van

Krieken & te Velde, 1992). In addition, considering the significant neural and hormonal control of splenic function, it is questionable whether surgically removed spleens provide a viable preparation; in studying splenic circulation *in vitro,* one might miss important neural influences. Previous investigators have used either radioactive microspheres or ultrasonic crystal techniques to measure organ blood volume or organ thickness respectively. Microspheres provide a good method in most cases to estimate organ blood flow. However, there are reports showing the existence of fast arterio-venous pathways or even arteriovenous shunts in the spleen (Levesque & Groom, 1981). Therefore, a proportion of injected microspheres passes right through these arterio-venous fast pathways or arteriovenous shunts. Thus, measurement of microspheres entrapped in the capillaries of the organ results in artifactually low estimates of total organ blood flow. With the ultrasonic crystal technique, instead of directly measuring the spleen blood flow, the organ thickness is measured. In this case, one must be extremely careful to ensure that the crystals are securely attached to the organ. Otherwise, when the organ contracts, the crystals might become detached.

Another critical parameter for investigating splenic function is the physiological condition of the animal during the experiment. Anesthetic effects on cardiovascular performance, and neural and hormonal function have to be taken into account; even lymphatic pumping is suppressed by anesthetic agents (Drake & Gabel, 1991). High sympathetic tone during the invasive surgery and increases of vasopressin levels would be expected to alter splenic circulation.

The first aim of this study was to establish the rat as a valid model for measuring splenic blood flow *directly* and *simultaneously*. There are obvious ethical and scientific reasons for the establishment of a chronic animal model which manifests similar changes in cardiovascular parameters to those observed in human. Establishment of such a model facilitates research on the mechanisms involved in the spleen's physiological and/or pathophysiological role in cardiovascular regulatory function. The use of conscious, recovered and unrestrained chronically-instrumented animals is of importance since cardiovascular hemodynamics are particularly susceptible to surgical trauma and the effects of anesthesia. This is of particular importance with regard to the sympathetic system and its critical involvement in modulating cardiovascular function in the hemorrhaged animal.

The use of ultrasonic transit-time flow probes has allowed investigation of splenic hemodynamic changes more accurately than had previously been possible. These studies are the first study to measure the splenic afferent and efferent blood flow in the intact conscious rat *directly* and *simultaneously* with these flow probes. The results presented in chapter 2, showing that both splenic afferent and efferent blood flow volume are much higher than previously reported, are of great significance. Other investigators have reported splenic blood flow to be about 2 ml/min in anesthetized animals using radioactive microspheres. This is close to the flow observed in this present study on the day of surgery. (Splenic arterial blood flow was 3.8 ml/min and splenic venous blood flow was 3.2 ml/min.) However, both splenic arterial and venous blood flow volume

increased dramatically as animal recovered from surgery. On day 10 post surgery, mean splenic blood flow was over 8 ml/min, with transient increases to as high as 13 ml/min. These results lend support to the growing body of evidence that anesthesia and invasive surgery effects cannot be ignored when cardiovascular function is studied. It should also be emphasized that, since this study allowed each rat to become familiar with the metabolism cage for at least 24 hour before running the experiment, and since the rats were kept in a quiet room throughout the experiment, artifacts due to stress were minimized. In this way, the results provide a clearer indication of the normal physiological situation than any previous experiments, where anesthetized, recently anesthetized plus surgery, and/or restrained animals were used.

In order to further investigate the relationship between anesthesia effects and surgical effects on splenic function, the first study of this thesis tested the response of injecting 20 mg/kg i.v. sodium pentobarbital on day 5 post-surgery. There were transient falls in the splenic arterial and venous blood flows, splenic arterial-venous difference and mean arterial blood pressure. Splenic arterial-venous differential blood flow returned towards control levels within 20 minutes despite significant depression of the mean arterial pressure for the entire experimental period, i.e. although anesthesia alone caused a transient fall in splenic blood flow, there was no significant change in the arterio-venous difference of blood flow. Moreover, despite depression of mean arterial pressure (86 mmHg) in response to administration of anesthesia, splenic blood flow and fluid extraction were significantly higher than the values of splenic circulation when mean

arterial blood pressure was 99 mmHg on the day surgery. It was concluded that it was the surgery not the anesthetic, that markedly depressed splenic circulation and fluid efflux function on day 0, and that fluid efflux was not correlated with MAP.

In response to hemorrhagic hypotension, splenic blood flow increased towards the pre-hemorrhagic levels within 10 minutes, and then remained stable throughout the entire experiment, despite the fact that mean arterial blood pressure was still significantly lower than in the control group. This lack of correlation between arterial blood pressure and splenic blood flow, and between mean arterial pressure and splenic fluid extraction, suggests that splenic hemodynamics are influenced by factors other than splenic perfusion pressure.

Whether there is an autoregulatory mechanism in the splenic circulation is still disputable. This study showed that, as is the case for the renal circulation, blood flow to the spleen is extremely high per gram weight of tissue. Progressive hemorrhage-induced changes, such as tissue ischemia and cellular dysfunction in multiple organs, are still major causes of morbidity and mortality. It suggests that, in order to maintain efficient splenic immunological defense during hemorrhagic hypovolemia, autoregulation is probably necessary to ensure that the splenic circulation and splenic function are maintained.

This present results also provide quantitative proof for the existence of a splenic deep lymphatic system. This is the first time that the splenic afferent and efferent blood flow have been measured and computed simultaneously in conscious animals. The results indicate that, as the animals recovered from surgery, the differential between splenic arterial and venous blood flow increased so that, at day 10 post surgery, only 70 - 75 % of the fluid entering the splenic circulation, left in the splenic venous effluent. With other reports now showing that there is structural evidence for a splenic deep lymphatic system, this study concludes that this persisting difference between inflow and outflow of splenic blood flow points to the existence of intrasplenic filtration. Since the huge volumes of extracted fluid (2.5 ml/min) cannot possibly be accommodated within the spleen, it is reasonable to suggest that lymphatic fluid is continuously drained into the lymphatic system. This hypothesis is further supported by the previous observation that the dye, Evan's blue, appears in the splenic lymphatic duct after injection into the splenic artery (Kaufman & Deng, 1993).

Attention was turned towards considering the fate of the lymphatic fluid draining from the spleen. Lymphatic fluid from the splanchnic circulation generally flows through a series of ducts, ultimately reaching the thoracic duct. By contrast, much of the lymphatic fluid from the spleen drains into a duct that taps into the hepatic portal vein (Job, 1915). It had been noticed that if the hepatic portal vein was partially occluded, lymphatic fluid could be seen to back up into the splenic vascular arcade (Unpublished observation). The two surfaces of the mesentery enveloping the vascular arcade supplying

the spleen became separated by a clear gelatinous substance. This same response has been observed after volume loading (Kaufman & Deng, 1993). It is possible that the spleen has this accessory lympho-venous tap to accommodate the very high rate of lymph flow from the organ.

These presently-reported studies demonstrate that, under physiological conditions, there is intrasplenic filtration. In response to expansion of the intravascular space, there is an increase in filtration of red cell-free, isooncotic fluid out of the circulating blood and into the lymphatic system. Therefore, the increase in hematocrit of the splenic venous blood is due to this fluid being *removed* from the splenic blood, not by the addition of enriched RBC from intrasplenic stores. It is believed that the spleen might be another component which regulates the circulating blood volume. In additi and behavioral mechanisms which conserve and replenish bodv nic filtration mechanism could be very important for *redistri* n response to hypo- or hypervolemia.

In response to hemorrhage, there are integrated neural, hormonal and behavioral responses whereby fluid losses are minimized, fluid intake is increased, and cardiac output and blood pressure are maintained so as to prevent the organism from falling into hemorrhagic shock. The lymphatic system is also considered to play an important role in self-resuscitation (Zhang, 1991). Therefore, it is possible that, during hemorrhage,

intrasplenic filtration of lymph is reduced, and fluid pooled within the lymphatic system is restored to the circulation.

The results from the hemorrhage experiment showed that, during hemorrhage (1.5% body weight), both the splenic afferent and efferent blood flows decrease. The differential between splenic arterial and venous blood flow was also abolished. This decrease of splenic arterio-venous difference was interpreted as the decrease of intrasplenic filtration of intravascular fluid out of blood into lymphatic ducts. Since this hemorrhage study did not maintain a constant level of hypotension for the entire experiment, there was only a transient decrease of splenic blood flow, followed by gradual recovery *towards* the pre-hemorrhage level within 10 minute after bleeding.

Some possibilities had been put forth to explain the reduction in fluid efflux in response to hemorrhage. Increased sympathetic stimulation of the lymph ducts could have raised lymphatic pressure thus impeding lymph formation. Sympathetic stimulation would also have caused blood to flow through splenic arterio-venous shunts, thus bypassing the sites of intrasplenic filtration (Levesque & Groom, 1981). Finally, sympathetic stimulation would have raised portal pressure thus impeding lymph drainage and, impeding splenic lymph formation upstream (Drake & Gabel, 1991).

Although the above results suggested that splenic lymph flow decreased in response to hemorrhage, there was still uncertainty about the mechanisms underlying translocation of protein-rich lymph from the interstitial/lymphatic compartments back to the intravascular compartment. However, Cope and Litwin (1962) have pointed out that the flow of thoracic duct lymph increases sharply, albeit transiently, following a severe, but non-lethal, hemorrhage. It is obvious that careful studies are needed on the role of the lymphatic circulation in self-resuscitation mechanisms during the early stage of the hemorrhage.

Another interesting new aspect about the role of the spleen in the regulatory mechanism has recently been noted. Studies have shown that portal hypertension in humans, is the exacerbated by splenectomy (Loftus et al, 1993; Ohta et al, 1992). In the rat, portal pressure has also been shown to *increase* after splenectomy, but to *decrease* after ligation of the splenic artery (Lin & Shan, 1992). This is probably due to the fact that intrasplenic filtration of fluid from the intravascular space into the lymphatic system is abolished by splenectomy. By contrast, retrograde perfusion of the spleen from the hypertensive portal vein would allow continued formation of lymphatic fluid and relief of pressure overload in the splenic artery-ligated animal. This idea that the spleen acts as one of the regulators in redistribution of body fluid is new, and further research is needed to more fully elaborate its function.

In summary, it is concluded that:

1. basal splenic blood flow in the fully recovered and conscious rats is considerably higher than previously reported;
2. splenic arterial inflow is consistently and significantly higher than splenic venous outflow in fully recovered and conscious rats;
3. up to 30% of fluid flowing into the spleen is removed from the circulating blood into the lymphatic system;
4. anesthesia itself has no sustained effect on fluid efflux; however, surgery markedly depresses blood flow and abolishes fluid efflux;
5. hemorrhage transiently reduces the arterio-venous difference of the splenic blood flows, intrasplenic filtration of plasma out of the blood and into the lymphatic system is thus attenuated during hemorrhage;
6. there is no simple relationship between splenic blood flow, fluid efflux and mean arterial blood pressure.

This study is the first to suggest that the spleen could possibly play a role in controlling intravascular volume by mechanisms other as a blood reservoir. The results have shown that the spleen is a gateway for the movement of fluid from the intravascular to the lymphatic compartments of the body. Recent experiments from this laboratory also point to the existence of a spleen-derived factor that appears to influence blood pressure

and renal output (Unpublished observation). These results contribute to increase understanding of splenic regulatory function under physiological conditions. Studies of splenic function under pathophysiological conditions such as portal hypertension await both researchers and clinical physicians' attention, and should shed light on some of the problems encountered by splenectomized patients.

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APPENDIX

APPENDIX A

SURGICAL PROCEDURE FOR IMPLANTATION OF CHRONIC ARTERIAL AND VENOUS CANNULAE

APPENDIX B

SURGICAL PROCEDURE FOR IMPLANTATION OF THE TRANSONIC FLOW PROBES

APPENDIX C

THEORY OF APPLYING ULTRASONIC TRANSIT TIME FLOWMETER AND FLOW PROBES

APPENDIX A

Surgical procedure for implantation of chronic arterial and venous cannulae:

All surgical instruments were sterilized with an Omni-Clave sterilizer for at least 20 min. Chronic femoral arterial and venous cannulae were prepared and sterilized by gas sterilization (University of Alberta Hospital Central Sterilizing Service) in advance then stored in sterile packages until required. For the femoral arterial cannula, one end of the 30 cm long of Micro-Renethane (Braintree, 0.30 mm ID, 0.64 mm OD) was pulled over a steaming kettle to slightly narrow the diameter. The narrow end of the cannula was bluntly beveled with sharp scalpel. A 3 mm long cuff of Silastic tubing (Dow Corning, 0.51 mm ID, 0.94 mm OD) was slipped over the beveled end of the cannula and positioned about 4 cm from the end. The femoral venous cannula was similarly prepared except that Silastic tubing (Dow Corning, 0.51 mm ID, 0.94 mm OD) was used. Before wrapping and sending the cannulae for sterilization, both the inner and outer surfaces of the tubing were washed with saline to ensure that no powder or dust remained on the cannulae.

On the day of surgery, we anesthetized the rat with sodium pentobarbital (Somnotol 60 mg/kg body weight, I.P.). Supplementary doses were administered as necessary during the surgery. Each rat was also administered a standard dose of Atropine Sulphate (0.2 ml, S.C.), and 0.22 ml Dihydrostreptomycin (Derapen-C, 200,000 IU

penicillin-G/ml, I.M.). Following surgery, Buprenorphine (0.02 mg/kg body weight, I.M.) was administered intramuscularly every 8 hours for 2 days.

The left medial surface of the femoral area (about 2 cm x 2 cm) and the nape of the neck (about 2.5 cm x 2.5 cm) were shaved and prepared for the femoral cannulae and attachment site of the cannulae. Betadine was applied to the surgical area to ensure sterility during surgery. The rat was placed in dorsal recumbency on the surgical table and wrapped with a piece of incise drape (Steri-Drape) all over the body. Its four limbs were restrained with tapes. Body temperature was measured rectally and maintained at 37°C by a heating pad. The rat was draped, leaving only the shaved parts exposed. The surgeon then donned a gown, mask and sterile surgical gloves.

With a sterile No. 15 scalpel blade, a 1.5 cm long skin incision was made on the medial aspect of the upper leg. Metzenbaum scissors were then used to enlarge the muscle wall incision and bluntly dissect the adductor longus muscles. Two flat retractors were positioned to separate the muscles, thus exposing the femoral artery, femoral vein and femoral nerve. With Moria forceps, the femoral nerves, artery and vein were carefully dissected. Two anchoring sutures were placed in the adductor longus muscle approximately 3 mm apart adjacent to the femoral vein. A 4-0 silk was placed around the femoral arterial vessel; bleeding would then be controlled by applying tension to the thread. Both cannulae were connected to 1 ml syringes filled with heparinized saline (10,000 units/litre). The arterial cannula was then measured on the surface of the rat and

the distance estimated so that the cannula tip would ultimately lie at approximately "belly button" level, i.e. just below the renal arteries and veins. Iris forceps were used to grasp the femoral artery, and a one-third cut was made with Vannas spring scissors at an approximately 45 degree to the vessel wall. The cannula was slipped in the artery and slowly advanced until it passed the confluence of the common iliac arteries. A pulse would then be detected in the cannula once the insertion was completed. Similar surgical procedures was used for inserting the femoral vein, the only difference being that no thread was needed around the vein to stop bleeding. Also, rather than cutting the vessels, the cannula was instead introduced through a puncture wound in the vessel wall made using a 23-gauge needle. Blood loss was minimized by lifting up on the wall of vessel during insertion. The femoral venous cannula was inserted to about 6 cm, i.e. it was advanced into the abdominal inferior vena cava (IVC). After the cannulations were completed, the ligatures on the adductor longus muscle were used to anchor those cannulae on either side of the Silastic cuffs.

Using a silver probe, the cannulae were drawn subcutaneously from the ventral surface of the leg, posteriorly around leg to the dorsal surface and dorsally to the back of the neck. The lines were then exteriorized and hooked up to "L"-shaped pieces of stainless steel tubing anchored with dental acrylic to a pedestal made from polypropylene monofilament sheeting. The other ends of the steel tubing were brought through the skin between the ears. The base of the pedestal lay flat against the muscle wall. To avoid tangling and kinking of the lines, loops of slack were made under the skin of the leg and

in a posterior pocket at the neck. Each cannula was anchored to the steel tubing with a 4-0 prolene ligature. The neck incision was then approximated using interrupted sutures in 4-0 prolene. The incision on the leg was closed using interrupted 3-0 silk sutures.

The femoral venous cannula was checked for patency, flushed, filled with approximately 200 µl of heparinized saline (10,000 units/litre), and sealed with a Silastic cap. The femoral arterial cannula was filled with about 20 µl of polyvinylpyrrolidone solution (8 mg PVP/10 ml heparinized saline) to prevent the arterial blood from forcing its way into the cannula and coagulating, and to retard blood flow up the cannula when handling the line in the recovered, conscious animal. This PVP solution was injected into the arterial line, not only after completion of surgery, but also after each use. All these cannulae could be left untouched for up to 10 days at a time without affecting patency. The femoral arterial cannula was used to monitor blood pressure during the experiment. The femoral venous cannula was used for injecting the test drug or hemorrhaging the animals.

The above surgical procedure would be completed smoothly within 45 min. According to my experience, if the femoral cannulae were inserted far enough into the abdomen vesseis, above the confluence of iliac vessels, those cannulae remained patent for at least 10 days. Femoral cannulation is an ideal experimental design for chronic cannulae and especially good for those experiments in which one does not wish to disturb the abdominal organs.

APPENDIX B

Surgical procedure for implantation of the transonic flow probes:

In preparation for surgery, all the instruments were sterilized with Omni-Clave sterilizer for at least 20 min. Two flow probes (1 mm R-series back cable exit with J reflector) were glued on a piece of Silastic sheet to stabilize probe position (Figure 1). They were connected to the T206 Small Animal Flowmeter, and signal amplitudes were verified to be above 1 Volt by pressing the "TEST" mode button on the meter. The pair of flow probes with two flange style of saddleback cuffs were then packed and sterilized with ethylene oxide gas. (University of Alberta Hospital Central Sterilize Service)

The rat was anesthetized with sodium pentobarbital (Somnotol 60 mg/kg body weight, I.P.). Supplementary doses were administered whenever necessary during the surgery. Each rat was administered a standard dose of Atropine Sulphate (0.2 ml, S.C.), and Dihydrostreptomycin. (Derapen-C, 200,000 IU penicillin-G/ml, I.M.) In order to inhibit the pancreatic enzyme secretion, a standard dose 0.3 ml of Aprotinin (Trasylol) was injected through the femoral vein cannula. Following surgery, Buprenorphine (0.02 mg/kg body weight, I.M.) was administered every 8 hours for 2 days.

The nape of the neck and abdomen were shaved and the surgical sites were cleaned with Providone iodine scrub solution. The rat was then wrapped with a piece of incise drape (Steri-Drape) all over the body and the rat placed in dorsal recumbency.

During surgery the rat was taped over an isothermal pad to maintain body temperature at about 37°C. The rat was draped leaving only the surgical area exposed.

A ventral midline abdominal skin incision was made with a sterile No.15 scalpel blade from the xiphisternum to the lower part of abdomen. The linea alba was located and lifted with Adson forceps, a puncture wound was made using the scalpel, and the abdominal incision was extended from the lower part of abdomen to the xiphisternum along the linea alba. The stomach was retracted out of the abdominal cavity with two wet Q-tips and wrapped in saline soaked 4 x 4 inch gauze, reflected up and laid on the thorax of the rat. The greater omentum, the point of attachment of the spleen, was removed so as to expose the splenic pedicle. The spleen could then be mobilized by dividing the ligaments extending between spleen-diaphragm and spleen-stomach. The vessels in the gastrosplenic ligament i.e. short gastric vessels, and the vessels to the body and tail of the pancreas of the spleen were ligated and divided. Thus, the primary splenic artery and vein in the splenic pedicle were the only remaining vessels to the spleen. The splenic lymph nodes on the top of the splenic vessels were gently moved aside, and the splenic artery and vein were exposed and bluntly dissected with wet Q-tips. Great care was taken in clearing the pancreatic and fatty tissue from the splenic artery and vein. With Moria forceps, the splenic artery was bluntly dissected from the splenic vein for a distance of about 5 mm. The two probes, glued on to the Silastic sheet, were placed under the splenic vascular arcade, and the splenic artery and vein slipped into individual probe windows. The probes' position was adjusted so that the vessels were centered within the windows

and the brackets did not tug on the tissue. Since the splenic vessels were extremely prone to vasoconstriction, care was taken to handle them as little as possible. In addition, a few drops of Xylocaine (Lidocaine 10 mg/ml) were applied to the area to dilate the vessels and improve the blood supply to spleen. A 1 cc syringe was loaded with sterile lubricating jelly (HR, Carter Products NY), care being taken to avoid the formation of air bubbles. Jelly was then introduced into the probe's acoustic window adjacent to the vessels. This HR lubricating jelly provided good acoustic coupling between vessels and flow probes. The stomach was then replaced into its original position over the splenic vessels and the flow probes.

Proper coupling and signal amplitude were verified by observing the diagnostic messages of the flowmeter in "TEST" mode ("1-Gd" on the digital panel and indication that probe was receiving a signal strength exceeding 60 %). A low signal strength reading "1-Lo" or an acoustic error "Er Acc" signal could usually be traced to an insufficient amount of lubricating jelly so that air bubbles and/or fat particles were in the acoustic windows. Generally, fibrous encapsulation of the probes within the first week of implant yielded a stable, air bubble-free coupling. The cables of the probe were anchored to each side of the abdominal muscle.

With a pair of fine scissors, a small puncture is made on each side of the abdominal wall corresponding to the level of the cables of the probes. A subcutaneous tunnel was created with curved Metzenbaum surgical scissors from the abdomen and up

to the nape of the neck. After passing through the holes on the abdominal wall, the CM4 connectors were tied to the eye of a silver probe and exteriorized to the neck of the rat. The CM4 connectors were placed in the saddleback cuffs and then sutured to the neck muscle cranial to scapulae, so that the connectors were stabilized in the position for external use. With the removable flat protective cap of the connectors, repeated chronic measurement of the splenic blood flows was thus feasible.

There were several critical points in this procedure for implantation of the flow probes and monitoring of splenic blood flow in a chronic preparation. Firstly, the splenic vessels had to be bluntly dissected freely so that there are enough space for implanting the probes. It was imperative to remove all fatty tissue in the probes' acoustic pathway. However, since these vessels were extremely sensitive to being handled, cleaning of the vessels had to be very gentle, attention being paid to ensure that they were not deflected from their natural course. Secondly, it was important to ensure that the flow probes be implanted and secured in position so as to maintain their proper alignment with the vessels without impeding the flow.

Ultrasonic transit time chronic blood flow measurement technique is a breakthrough for the hemodynamic studies in research. It allows the researcher to study biological functions under natural physiological condition. It is well known that the effects of anesthesia on cardiovascular parameters have to be taken into account in studies of cardiovascular physiology. These probes allowed us to measure the splenic

hemodynamic changes directly and repeatedly in the conscious rats. These techniques allowed us, for the first time, to accurately measure splenic blood flow. These are also the first reports of simultaneous estimation of afferent and efferent blood flow to an organ.

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APPENDIX C

Theory of applying Ultrasonic Transit Time Flowmeter and Flowprobes:

In order to investigate the splenic hemodynamic changes under a variety of experimental conditions, an ideal method for measuring splenic arterial blood flow and splenic venous blood flow *directly, simultaneously and chronically* needed to be developed. Transonic Systems' Animal Research T206 dual channels Flowmeter (Figure 1), which utilizes transit-time principles of ultrasound, was used directly to measure volume flow in the splenic artery and vein. This small animal flowmeter is equipped with analog and digital displays to monitor volume flow and ultrasonic signal coupling. Therefore, both splenic arterial and venous pulsatile and average flow signals are simultaneously accessible via analog outputs. With the personal computer interface option, consisting of A/D converter circuitry and FLOWTRACE software connecting the flowmeter to a standard IBM-PC computer via its serial RS-232 port, digitized flow information with 12 bits of resolution at 100 samples per second was sent to the VGA-equipped computer. In addition, the pressure option (an amplifier for bridge type pressure transducers), allowed instantaneous blood pressure data to be recorded in IBM-PC computer at the same time. Thus, this unique technical set up enabled us to obtain a true physiological picture of splenic circulation and splenic hemodynamic changes under various conditions. The absolute values of splenic arterial and venous blood flow per minute, arterial blood pressure with diastolic and systolic pulsatile, values of MAP, the changes in splenic blood flow corresponding to the changes of arterial blood pressure,

and the effects of anesthesia drug and hemorrhage on splenic blood flow were displayed, recorded, stored and retrieved by an IBM-PC compatible computer. Most importantly, this ultrasonic transit-time flowmeter and flowprobe design allowed us make repeated studies of cardiovascular physiological function in fully recovered, conscious and unrestrained animals. FLOWTRACE software allowed us to digitally record the experiment on diskette for future graphical display and data analysis.

An ultrasonic transit-time flowprobe (Figure 2) consists of a probe body with two ultrasonic transducers and a fixed acoustic reflector. The ultrasonic transducers are positioned on one side of the vessel under study and the acoustic reflector is positioned midway between these two transducers on the opposite side of the vessel, so that the upstream and downstream ultrasonic signals can be transmitted through the vessel and between the transducers. Our 1 mm R-Series J-reflector bracket probes, designed for 0.5 mm to 1.0 mm O.D. vessel in chronic application, have the transducers positioned at a 45° angle to the vessel under study with higher resolution and accuracy. Its absolute accuracy percentage is ± 10% and relative accuracy percentage is ± 2%. Two identical 1R flowprobes were glued together into position on a sheet of Silastic, the splenic arterial afferent blood flow volume and splenic venous efferent blood flow volume could be recorded at the same time.

Each flowprobe integrates the upstream and downstream transit-time of the reflective beams twice on its reflective pathway. During the downstream transit-time

measurement cycle, a planar wave of ultrasonic signal, which was emitted by the upstream transducer under electrical excitation intersects the vessel in the downstream direction. This wave then bounces off the "acoustic reflector", intersects the vessel again and is received by the downstream transducer where it is converted into electrical signals. The same mechanism happens in the upstream transit-time measurement cycle but in a reversed direction. Although there is an increase in total-transit time in upstream cycle due to the sound wave travelling against the flow direction, the total transit-time during the downstream cycle decreases due to the wave travelling with the flow. The flowmeter then derives an accurate measure of the "transit time" it took for the wave of ultrasound to travel from one transducer to the other, and the difference between the upstream and downstream transit time is integrated as the measurement of volume flow. With these two intersections, the transit-time of the acoustic signal across the full area of the vessel determines the volume flow rather than velocity. Furthermore, readings are automatically compensated in case of misalignment of the probe on the vessel. Measurements are thus largely insensitive to flow velocity profiles, vessel-probe alignment, motion artifacts, hematocrit, and electrical noise. Streamlined CM4 connectors, which is mini 4-pin sealed connector with separate ROM key, enabled us to make repetitive measurement of splenic arterial and venous blood flow in chronically implantated animals. During the measurement of the flow volume, each probe was used with its serial numbered coding key for specific accuracy performance.

The pressure interface amplifies the microvolt signal from an Abbott "Transpack" external disposable pressure transducer to a voltage that ranges between -5V and +5V. This pressure signal accompanies the flow signal via the RS-232 cable to the computer. With the 5 volt excitation, the resultant sensitivity is 80 mmHg per volt of amplifier output. Therefore, the diastolic and systolic of the arterial blood pressure pulsatile are displayed on the computer screen; mean arterial blood pressure was also be recorded for later analysis.

We used two channels for measuring the splenic blood flows, and one for measuring the mean arterial blood pressure. The blood flow channels were calibrated using the pre-set calibration factors from the ultrasonic probes. Calibration of the pressure channel was done using a pressure manometer to provide a reference pressure of 100 torr. These pressure channels calibration remains relatively constant if the same pressure transducer is used. No on-site calibration of the flow probes was necessary since each probe was factory precalibrated to meet its stated specification before being supplied to the customers. However, before applying the flow probes to the living vessels, they needed to be verified for proper function using the diagnostic features on the flowmeter. In the saline bath, the probe gave at least 1.0 ± 10% ultrasonic signal strength on the analog meter when its ultrasonic sensing window was clean and no air bubbles were present in the window. During the measurement of the blood flow volume, proper coupling and signal amplitude were verified by observing the meter's diagnostic messages in "TEST" mode. It was imperative that, on the digital panel meter, the "1-Gd"

was displayed and the receiving signal strength on the analog panel meter indicated of exceeding 60% i.e. in the acute experiment the reading exceeded 0.6. A low signal strength reading "1-Lo" or an acoustic error "Er Acc" signal could usually be traced to an insufficient amount of lubricating jelly so that air bubbles and/or fat particles were in the acoustic windows. The "√ TEST" light illuminated when the flow signals recorded by the flowmeter were probably below the accuracy specifications. Coagulated blood and HR lubricating jelly are recommended couplants, providing good acoustic coupling between vessel and flowprobes. During chronic implantation of the probes, the ultrasonic signal strength improves due to fibrous encapsulation of the probe which yields a stable bubble-free coupling. We found, as have other reserchers, that the recovery period is very critical because it allows the fibrotic build-up surrounding the probe's acoustic window which ensures more accurate measurement of the volume flow. It also eliminates the effect of surgery and anesthesia on volume flow.

Ultrasonic Transit-Time flowmeter and flowprobes enabled us to complete our study under physiological condition with high accuracy. We therefore were able :

1. to measure the splenic blood volume flow directly;
2. to simultaneously compute splenic afferent and efferent volume flows;
3. to avoid anesthesia and surgical effects on the cardiovascular system;
4. to repetitively measure flows over the long-term;
5. to enable vessels be fully functional for flow measurement in a non-constrictive and non-occlusive design.

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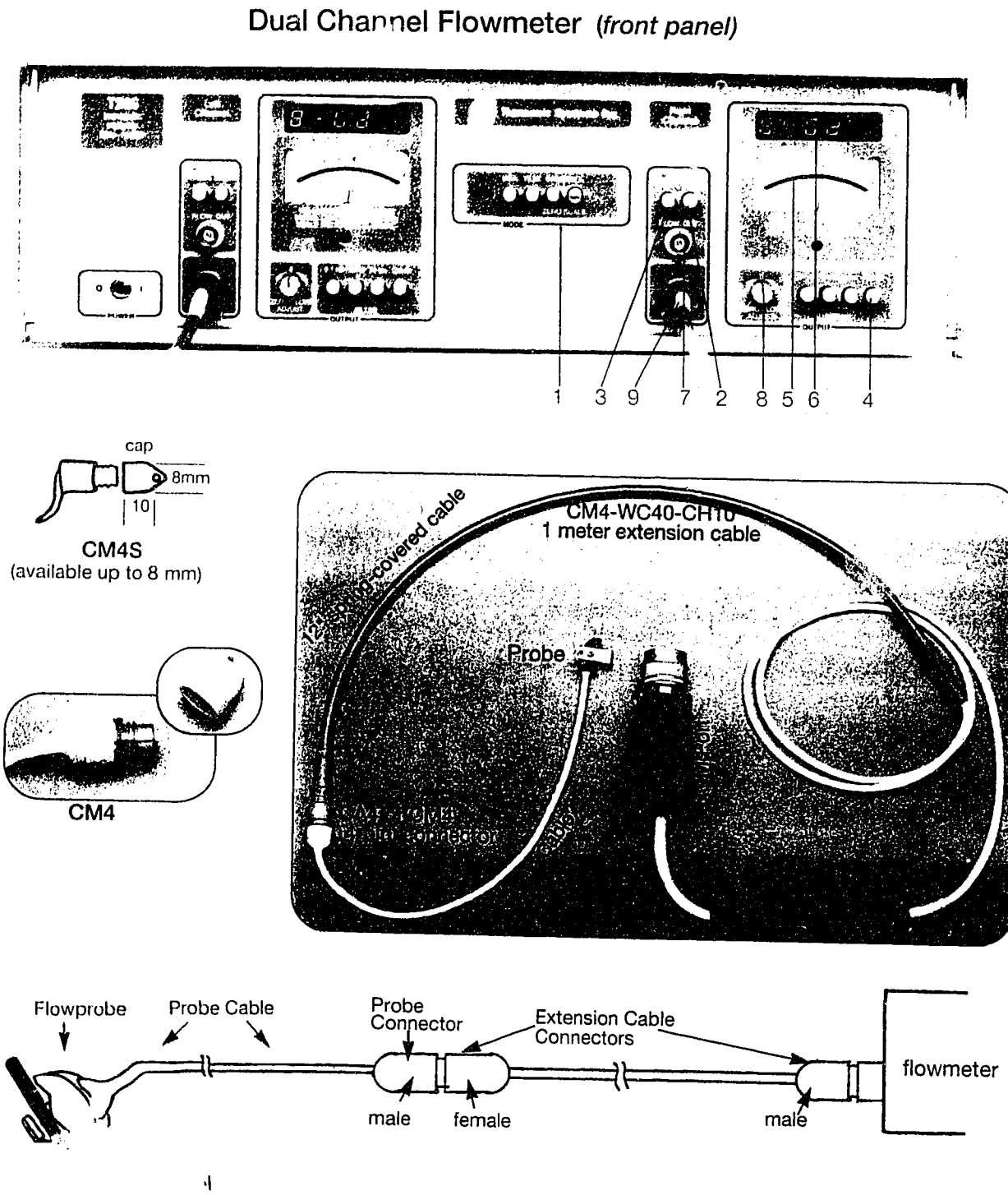

Figure 1. Transonic T206 dual channel Small Animal Flowmeter and accessories.

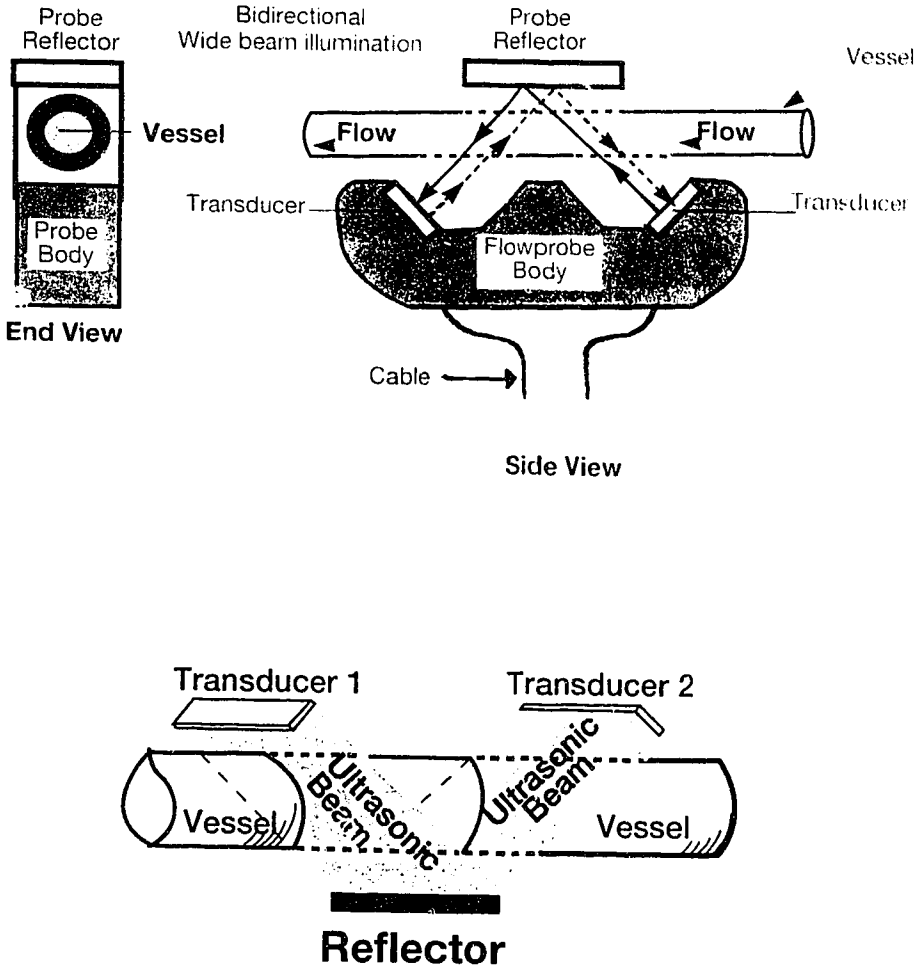

Figure 2. Ultrasonic transit-time flowprobe. Schematic views of the perivascular Transonic ultrasonic volume flowsensor. The entire blood vessel is fully and evenly placed within a beam. The transit time of the wide beam, two transducers pass ultrasonic signals back and forth, alternately intersecting the flowing liquid in upstream and downstream directions. The difference between the upstream and downstream integrated transit times is a measure of volume flow, independent of vessel dimensions.

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