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INNERVATION AND VASCULAR PHARMACODYNAMICS OF THE MOUSE SPLEEN

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Abstract

Neurohistochemical and *in vivo* and electron microscopic methods demonstrated α-and β-adrenergic receptors and adrenergic innervation in arterioles and "arterial" capillaries of the mouse spleen. Such innervation and receptors in venules and channels within the red pulp were sparse. Cholinergic innervation and receptors were judged to be absent in the microvasculature. Histamine elicited arteriolar dilation which was blocked by metiamide suggesting the presence of H_2 receptors. However, following blockade of H_2 receptors, histamine produced arteriolar constriction. Serotonin elicited only venular constriction. Lactic acid caused arteriolar constriction; bradykinin and prostaglandins (PG) E_2 and $PGF_{2\alpha}$ triggered arteriolar constriction, but only at higher concentrations. The vasoconstriction evoked by cholinergic agonists, histamine, lactic acid, or PGs was partially or completely antagonized by α-adrenoceptor blockade or by reserpine, and the vasoconstrictor responses to histamine, lactic acid, PGs, bradykinin were enhanced in the presence of functional adrenergic nerves. In the latter case higher doses of phentolamine provoked arteriolar vasospasm. Although adenine nucleotides, guanosine, inosine, sodium phosphate, and sodium chloride elicited no response, adenosine was a potent vasodilator. This dilation was not blocked by β-adrenergic antagonists, and it was enhanced in the presence of functional adrenergic nerves. The data suggest that: (1) cholinergic agonists, lactic acid, histamine, and PGE_2 and $PGF_{2\alpha}$ cause α-mediated arteriolar constriction by releasing stored neurotransmitter(s) from splenic nerves, and (2) subthreshold quantities of neurotransmitter(s) may modulate microvascular sensitivity to vasoactive agents which act directly upon the vascular wall.

Key Words: spleen, mice, microcirculation, neuroimmunomodulation, sympathetic nerves, parasympathetic nerves, sensory nerves, neurohumoral regulation, neurohumoral interaction, vascular pharmacodynamics.

Introduction

Most information about splenic microvascular regulation has been extrapolated from investigations of humoral effects on innervated vessels larger than 300 microns or from measurements of flow, resistance, and clearance following arterial or venous injections of vasoactive substances or tracers. These studies fail to elucidate directly the sensitivity of the microvascular system to these substances in intact innervated spleens, the discrete site(s) of chemical interaction within the "resistance" vessels of the spleen, or the role of hormones, the nervous system, or other stimuli (e.g., anesthesia), in direct and indirect microvascular responses. Indirect effects are induced by these chemicals: (a) modifying the responses of vascular and capsular smooth muscle to vasoactive agents, or (b) causing the release of stores of neurotransmitter(s) from splenic nerves.

The principal goal of the following literature review is to consider the effect(s) of potentially vasoactive substances and synthetic agonists, antagonists, or other inhibitors, on the capsule and vasculature of the mouse spleen. The anatomic distribution of autonomic and sensory nerves as well as the role of neural mechanisms in splenic function also are evaluated in order to explore the interaction of neurohumoral regulatory mechanisms following administration of different anesthetic agents. Where relevant, an assessment is made of the pathophysiologic significance of elevated levels of neurotransmitters, or other humoral agents, upon splenic blood flow in an attempt to elucidate the potential involvement of each in local regulation of blood flow during conditions of optimal circulation and of low-flow.

The current treatise is a comprehensive review of the author's original work on the mouse spleen and, therefore, reflects primarily his interests and his contributions to the field of research. The decision to restrict the focus of the manuscript to the murine spleen is predicated on the vast interspecies variations reported: (a) in vascular sensitivity and responsiveness to neurostimulation and neurohumoral agents, and (b) in nerve and receptor density and distribution. Intraspecies controversies also are well documented in the literature and they result, at least in part, from the application of different

experimental designs and protocols to investigations on splenic innervation and vascular pharmacodynamics. These discrepancies within the literature are adequately reviewed by Davies and Withrington [11] in 1973 and by Reilly [40] in 1985. However, references for relevant research since 1985 are provided for the canine, feline, rodent, guinea-pig, pig, bovine, rabbit, and murine spleens.

Autonomic and Sensory Innervation

Innervation of the mouse spleen appears to be relatively sparse. While adrenergic (sympathetic) nerves have been demonstrated using neurohistochemical and electron microscopic techniques, there is no evidence for cholinergic (parasympathetic) or sensory (afferent) innervation in this species [14, 40, 43, 44].

Adrenergic nerves are distributed to the smooth muscle of arteries and arterioles of the mouse spleen. The splenic capsule, veins, venules, and channels in the red pulp, lack morphologically demonstrable innervation. In addition, arteries in the white pulp, hilus, and trabeculae of the spleen are supplied by unmyelinated adrenergic nerves containing dense-cored vesicles in the mouse. The fluorescent product for formaldehyde histochemistry was apple-green in color, which is characteristic of the transmitter(s) norepinephrine and/or dopamine in these nerves. These results support physiologic and pharmacologic data suggesting functional (sympathetic) innervation of the arteriolar bed of the murine spleen [31, 40-42].

These findings are consistent with those reported for the spleens of rats [2, 8, 13-15, 35], rabbits [14], cats [7, 32, 33], and pigs [20, 22] where the sympathetic (postganglionic) fibers originate from neurons located in the celiac and superior mesenteric ganglia [2, 32]. However, unlike the murine spleen, myelinated afferent nerves have been demonstrated in the feline spleen [7, 33] and unmyelinated adrenergic (sympathetic) nerves in the splenic capsule, veins, and venules of a number of mammalian species including cats and pigs [20, 22, and for a comprehensive review prior to 1985 see, 11 and 40]. More recent studies suggest that non-adrenergic (sympathetic) nerves innervate the spleen, and that they and adrenergic (sympathetic) and sensory (afferent) nerves contain vasoactive neuropeptides in cat, dog, rabbit, rat, mouse, guinea-pig, pig, and bovine spleens [14, 17, 20-28, 36, 37, 46].

Neuropeptides exert pre- and post-junctional effects in the spleen which regulate norepinephrine release and vascular tone [14, 17, 20-28, 36, 37, 46]. For example, in the splenic artery of the pig, neuropeptide Y (NPY) and endothelin mediate their vasoconstrictor effects by direct stimulation of vascular smooth muscle independent of an endothelium-derived contracting factor [36]. In contrast, the vasorelaxation induced by substance P, but not that of vasoactive intestinal polypeptide (VIP) or calcitonin gene-related peptide (CGRP), is endothelium dependent in action [36]. Experimental release of norepinephrine and co-localized NPY is influenced by the pattern and frequency of adrenergic (sympathetic) nerve stimulation in cat and pig spleens [23-27, 37]. Activation of pre-junctional α-2 adrenoceptors inhibits the release of both norepinephrine and NPY from adrenergic (sympathetic) nerves supplying these spleens [24, 25, 46].

Innervation of Avascular Cellular Elements

In the mouse, some of the adrenergic (sympathetic) nerves are not contiguous with the vasculature of the spleen [14, 31, 40, 43, 44]. Unmyelinated terminal nerve fibers and endings are adjacent to reticular cells and developing lymphocytes within the periarteriolar lymphatic sheath (PALS) of the spleen [14, 31, 40, 44]. However, a functional relationship between such nerves and the reticulum, or blood elements, has yet to be established in the mouse spleen. Since reticular cells are suggested to be contractile [31, 40], they may participate along with the smooth muscle in the capsule and trabeculae in affecting the large decrease in splenic volume observed following adrenergic stimulation. Yet to be elucidated, is the role of resident cells in the spleen in secreting vasoactive compounds. Macrophages produce prostaglandins and hemopoietic elements (e.g., developing lymphocytes and/or erythrocytes) also may release these or other substances that influence intrasplenic blood flow [30, 40].

Reports by Bryon [4-6] and Coffey *et al.* [9] suggest that β_1 and α-adrenoceptors modulate the cell cycle of hematopoietic stem cells or lymphocytes. Catecholamines such as norepinephrine, epinephrine, or isoproterenol trigger stem cells from G_0 into DNA synthesis. In the mouse, depletion of norepinephrine upregulates the number of β-adrenoceptors distributed on lymphocytes, and upregulation is associated with impaired immunoresponsiveness to antigens *in vitro* [18].

A relationship is established in the rodent spleen for adrenergic (sympathetic) nerves and co-localized catecholamines and neuropeptides and for T and B lymphocytes and macrophages [8, 13-15]. The pattern of growth and development of these adrenergic (sympathetic) nerves seems to parallel age-dependent changes in T cell compartmentalization within PALS and to effect lymphocyte packing and immunocompetence [1]. These recent findings are exciting for they focus attention on the potential role of splenic adrenergic (sympathetic) nerves in the modulation of T and B lymphocyte trafficking, antigen capture and presentation, and T cell activation [13].

Microvascular Pharmacodynamics

Adrenergic Mechanisms

Pharmacologic and neurophysiologic studies of the spleen have been extensive [for a comprehensive review through 1985 see, 11 and 40]. In the mouse, norepinephrine, epinephrine, or isoproterenol, have been

demonstrated to be vasoactive, and constrictor α- and dilator β-receptors have been isolated for these chemicals [40-42].

In the rabbit and cat, splenic α-adrenoceptors are classified as the α-2 subtype [12] with their relative distribution being 75% α-2A and 25% α-2B [34]. However, adrenergic receptors in pigs are predominantly α-1 [22, 24]. In addition to these post-junctional α-adrenoceptors, pre-junctional β-2 receptors are localized on adrenergic (sympathetic) nerve terminals in cats [10], while in dogs these β-receptors are distributed post-junctionally on splenic arteries and veins and pre-junctionally on associated splenic nerve bundles [19].

In vivo microscopic studies indicate that arteries and arterioles of the splenic red and white pulps are the vascular segments responsive to pharmacologic or neural stimulation in the mouse [16, 29, 41, 42]. This includes the terminal segments of arterioles called "arterial" capillaries which supply the red pulp. "Arterial" capillaries have an internal diameter of 7 to 10 microns and are defined by a single layer of discontinuous smooth muscle surrounding their endothelium. While venules and veins do not respond following α- or β-adrenoceptor stimulation, some vasoactive substances have been suggested by McCuskey and co-workers to influence the permeability of the venous sinuses to blood cells [30, 31].

Cholinergic Mechanisms

Cholinergic (parasympathetic) innervation from the vagus nerve has been discounted in the mammalian spleen [2, 11, 40, 45]. The consensus of opinion is that vascular and capsular responses (i.e., constriction and contraction, respectively) after electrical stimulation of the vagus nerve, or after the systemic administration of cholinergic substances, are secondary to a reduction in cardiac output and mean systemic blood pressure.

Transillumination studies of the mouse spleen using high resolution *in vivo* microscopy reveal that cholinergic stimulation produces arterial and arteriolar constriction and decreased blood flow [16, 41]. The constriction of arterioles and "arterial" capillaries is antagonized by atropine which suggests a direct muscarinic action. However, in this species a nicotinic action cannot be ruled out, because α-antagonists also block microvascular responses to cholinergic agonists [40-42].

Vasoactive Amines

Serotonin has been reported to produce constriction of venules in the spleens of the mice evaluated by *in vivo* microscopic methods [40, 41]. In contrast, histamine elicits dilation of arterioles and "arterial" capillaries in the mouse spleen. This response is mediated via H_2 but not H_1 receptors [40-42]. However, following blockade of H_2 receptors, histamine also provokes constriction of these microvascular segments and capsular contraction [16, 40-42]. Since both responses are blocked by α-adrenergic antagonists and reserpine, it is suggested that they are mediated by the release of stored neurotransmitter(s) from adrenergic (sympathetic) nerves [42].

Nucleotides and Their Degradation Products, Ions, and Lactic Acid

In the spleen of most species, ATP, ADP, AMP, adenosine, guanosine, inosine, sodium and potassium chloride, inorganic phosphate, and lactic acid, have <u>not</u> been implicated in the local regulation of blood flow [40]. Of these constituents, only adenosine and lactic acid have been shown with *in vivo* microscopy to be vasoactive in the mouse spleen [40-42].

Adenosine evokes arteriolar dilation and increases both the linear velocity of blood flow and the number of channels with flow in the murine spleen [40-42]. These responses are not modified by β-adrenoceptor blockade and may be mediated by pre- and/or post-junctional adenosine receptors since activation of these receptors has been reported to elicit vasodilation [3, 38, 39].

In contrast, lactic acid causes α-mediated arteriolar constriction and reduced blood flow, and it promotes storage of blood in the red pulp of the mouse spleen [40-42]. These responses are suggested to be due to released neurotransmitter(s) from adrenergic (sympathetic) nerves, since they are abolished by α-blockers or by reserpine [42]. In higher doses (greater than 10 μg/ml), lactic acid elicits erratic arteriolar contractions which appear to be related to pH, because they are not inhibited by α-receptor blockers or by reserpine.

Prostaglandins (PGs)

PGE_2 or $PGF_{2\alpha}$ administration reduces blood flow and promotes storage of blood in the red pulp of the mouse spleen [40-42]. Arteriolar constriction also is provoked by each compound; however, this response is observed only at higher doses (100 μg/ml). Although the cumulative effects produced by the PGs are attenuated by α-adrenoceptor blockers, they are abolished by reserpine. Such antagonism suggests that PGE_2 or $PGF_{2\alpha}$ release stored neurotransmitter(s) from adrenergic (sympathetic) nerves in the mouse spleen [40-42].

Bradykinin

A direct action of bradykinin on vascular smooth muscle has been proposed in the mouse [40-42]. Results using *in vivo* microscopic methods suggest that bradykinin induces arteriolar constriction, increased storage of blood, and reduced blood flow through the red pulp. These responses in the mouse are not influenced by α-adrenoceptor blocking agents or by reserpine.

More recent research suggests that bradykinin stimulates splenic afferent nerves and initiates differential spleno-splenic reflexes which modify resistance and capacitive functions of the feline spleen [7, 33]. This interaction discharges splenic efferent nerves and increases splenic venous and systemic arterial pressures and heart rate.

Interaction of Neurohumoral Regulatory Mechanisms

The responses of the microvasculature to many vasoactive substances may differ depending upon the functional state of the nerves innervating these vessels. Therefore, the influence of the nervous system on the humoral regulation of the splenic microvasculature was evaluated using high resolution *in vivo* microscopic methods [31, 40, 42].

Mice were anesthetized with sodium pentobarbital (0.03 mg per g body weight [b.w.]) which results in electrically and pharmacologically excitable adrenergic (sympathetic) nerves. A variety of vasoactive substances were administered topically to the surface of the spleens of these mice, some of which had been pretreated with reserpine (intraperitoneal administration of 5 mg per kg b.w.). These data were compared with those from mice anesthetized with urethane (2.5 mg per g b.w.) [31, 40, 41]. At this concentration of urethane, neural influence on the splenic microvasculature is effectively removed thereby limiting the possible modification of microvascular responses by direct pharmacological stimulation of the splenic efferent nerves [42].

With functional adrenergic (sympathetic) innervation, there is a significant increase in the magnitude of arteriolar constriction elicited by histamine, prostaglandins (PGs) E_2 and $F_{2\alpha}$, lactic acid, and bradykinin, and of arteriolar dilatation produced by adenosine and isoproterenol. Phentolamine at higher concentrations causes arteriolar vasospasm. The response elicited by isoproterenol but not adenosine is antagonized by propranolol, while the constriction produced by all substances except phentolamine and bradykinin is blocked by an α-adrenergic antagonist. Responses to several of these substances are abolished by reserpine pretreatment.

These data suggest: (a) that lactic acid, histamine, and PGE_2 and $PGF_{2\alpha}$ cause α-mediated arteriolar constriction by releasing stored neurotransmitter(s) from adrenergic (sympathetic) nerves; (b) that subthreshold quantities of neurotransmitter(s) may modulate microvascular sensitivity to bradykinin, isoproterenol, adenosine, phentolamine, and PGE_2 and $PGF_{2\alpha}$ in the presence of responsive adrenergic (sympathetic) nerves; (c) that the anesthetic agents do not significantly alter the sensitivity of the contractile components of the splenic microvascular system to vasoactive substances which act directly upon the vascular wall.

Although the mechanism provoking arteriolar vasospasm to phentolamine is unresolved, blockade of prejunctional α-2 adrenoceptors in cats [21] and pigs [24] causes release of vasoconstrictor neuropeptides (e.g., neuropeptide Y) from splenic adrenergic (sympathetic) nerves. Alternatively, adenosine may reflexively trigger sympathetic discharge in the kidney and elevate circulating levels of angiotensin II [7]. It is conceivable that neuropeptide Y and/or angiotensin II mediate phentolamine-induced vasospasm in the murine spleen with functional splenic innervation.

Pathophysiologic Significance

Splenic blood flow is principally controlled by splenic arterial resistance in the mouse. Smooth muscle in the capsule and trabeculae contribute to the regulation of flow. Dynamic, moment-to-moment adjustments in muscular tone affect the rate(s) of filtration, synthesis, storage, and release of blood elements in the red and white pulps of the spleen. Vasoconstrictor mechanisms, primarily of an α-adrenergic origin, predominate and are relatively strong influences governing blood flow to and from, as well as within and between, the red and white pulps of the spleen. Vasodilator mechanisms are poorly developed in the murine spleen. These mechanisms are thought to be operative during conditions of optimal circulation (e.g., hyperemia, hematopoiesis) and of low flow (e.g., hemorrhage, shock, polycythemia, anemia, etc.). However, studies are required to establish that the putative vasoactive agents released in these conditions accumulate in quantities sufficient to modify splenic blood flow. Yet to be elucidated is the role of endogenous (vasoactive) neuropeptides in the regulation of splenic blood flow and hematopoiesis (e.g., erythropoiesis, lymphopoiesis, etc.) in the mouse.

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Discussion with Reviewers

J.G. Chamberlain: α-adrenergic blockade abolishes the difference in the hematocrit (Ht) of the red pulp and the splenic vein outflow. Can you postulate a mechanism based on your studies?

Author: Blockade of α-adrenoceptors in the spleen would be expected to modify vascular tone which in turn would provoke vasodilatation. Vasodilation in the murine spleen in response to adenosine or isoproterenol has been reported to increase both the linear velocity of blood flow, and the number of channels with flow, within the red pulp [40-42]. I believe this mechanism is responsible for purging red blood cells from the red pulp thereby abolishing the difference in Ht seen between the red pulp and splenic venous outflow following blockade.

J.G. Walmsley: What are the more recent relevant investigations related to this topic, particularly related to peptidergic innervation and the role of endothelium?

Author: Neuropeptides have been localized in adrenergic, non-adrenergic, and/or afferent nerves of the spleen in a number of mammalian species including the mouse [14, 17, 20-28, 36, 37, 46]. These peptides exert pre- and post-junctional effects in these spleens which regulate neurotransmitter release and vascular tone. To my knowledge of all the peptides isolated in the spleen, only substance P in the pig has been shown to be endothelium dependent in its action.

J.G. Walmsley: You have reviewed investigations which suggest a role for adrenergic nerves in the modulation of the cell cycle of hematopoietic stem cells or lymphocytes and modulation of T and B cell function. Considering the present interest in acquired immune deficiency related to HIV infection, does the author see any potential for pharmacologic alteration of splenic vascular neuroeffector mechanisms increasing the number of healthy T_4 cells or decreasing the replication (and HIV production) of infected T_4 cells?

Author: Although no direct evidence exists for a role of adrenergic (sympathetic) nerves in neuroimmunomodulation and vasoregulation in HIV infections, the studies reviewed herein do provide intriguing indirect support for the potential involvement of such nerves in immunocompetence and the local regulation of splenic blood flow. A relationship has been established for adrenergic nerves and for T and B lymphocytes and macrophages in rats and mice [1, 8, 13-15, 18]. Receptor-mediated adrenergic stimulation may enhance the phagocytosis of infected T_4 cells thereby inhibiting HIV replication. Furthermore, vascular neuroeffector mechanisms may facilitate such pivotal functions as T and B lymphocyte entry into the spleen, antigen capture in marginal zones, antigen presentation and activation of T cells in PALS and of B cells in splenic para-follicular and marginal zones, and lymphocyte egress in the outer marginal zone of the spleen [15].

J.G. Walmsley: What reasons could there be for adenosine and lactic acid being vasoactive in the mouse spleen and not so in most species along with other substances?

Author: Unlike most mammalian spleens, the murine spleen is both an erythropoietic as well as a lymphopoietic organ [30]. These authors postulate a role for adenosine in the functional hyperemia reported in the erythropoietically stimulated mouse spleen. As specialization in the red blood cell line proceeds, there is a gradual elevation of intracellular c-AMP levels. It is hypothesized that adenosine formed from the degradation of these elevated levels of c-AMP may elicit hyperemia 6-10 hours after erythropoietic induction [41]. Further-

more, since erythropoiesis is an energy-consuming process, the pathway by which ATP is metabolized to ADP and AMP also must be considered as an alternative mechanism leading to putative increases in local concentrations of the vasodilator adenosine.

The purported vasoactive properties of lactic acid are suggested in the erythropoietic red pulp of the mouse due to its vast population of erythrocytes which are dependent on anaerobic metabolism. Therefore, lactic acid also might accumulate in sufficient concentrations in the red pulp to modulate blood flow in conditions of low flow (e.g., hemorrhage, shock, polycythemia, anemia, etc.).

J.G. Walmsley: Through your many detailed studies you and your coworkers have concluded that the histamine contractile response is mediated through receptors on the sympathetic nerves. Are you suggesting that there are H_1 receptors on sympathetic nerves, or is there some other receptor-mediated event which is more consistent with your results?

Author: We have no data to support the existence of H_1 or H_2 receptors on sympathetic nerves in the murine spleen. As we have reported previously [41, 42], histamine-induced vasoconstriction is noted only after blockade (not stimulation) of H_2 (and not H_1) receptors, and only when sympathetic nerves are relatively unresponsive (hyporeactive) to electrical neurostimulation (urethane anesthesia). Our results do suggest, however, that histamine activates the release of norepinephrine from sympathetic nerves by some as yet undefined mechanism when splenic nerves are responsive (reactive) to electrical neurostimulation (pentobarbital anesthesia). This conclusion is based upon research demonstrating blockade of histamine-induced vasoconstriction by either phentolamine or by reserpine in mice anesthetized with pentobarbital.

H.H. Ditrich: You mention that catecholamines released through the adrenergic innervation may influence the cell cycle of hematopoietic cells. Could you comment if adrenergic innervation was found in other hemopoietic tissues like bone marrow and if an influence of these nerves on the development of the blood elements was found?

Author: Adrenergic innervation has been demonstrated in the bone marrow, thymus, lymph nodes, gut-associated lymphoid tissue, as well as the spleen in a variety of mammalian species [for a review see, 14]. Depletion of norepinephrine has been shown to upregulate the number of β-receptors on blood elements, and upregulation is associated with impaired immunoresponsiveness to antigens *in vitro* [18]. Therefore, it is postulated by these investigators that adrenergic nerves and neurotransmitters influence the development of blood elements in these tissues and organs.

H.H. Ditrich: You describe that adenosine produces vasodilation while other adenine nucleotides had no effect on local blood flow. Adenosine-induced vasodilation is not endothelium-dependent relaxant factor (EDRF) dependent while the vasodilation through adenine nucleotides is [Pohl *et al.*, 1987, Am. J. Physiol. 253: H234-H239]. Does this mean that EDRF dependent vasodilation does not exist in the mouse spleen and that this may explain the poorly developed vasodilator mechanisms in the spleen?

Author: I can cite no research which addresses this question in the spleen. However, the action of substance P (i.e., vasodilation) is suggested to be endothelium dependent in the pig spleen [36]. Since an endothelium dependent mechanism appears to be present (at least in the pig), I doubt that the absence of EDRF in the spleen can explain why vasodilator mechanisms are poorly developed in these species.

H.H. Ditrich: What mechanism causes the observed arteriolar vasospasm after high phentolamine concentrations?

Author: The mechanism provoking arteriolar vasospasm to phentolamine is unknown in the murine spleen. However, since blockade of prejunctional α-2 adrenoceptors in cats [21] and pigs [24] causes the release of vasoconstrictor neuropeptides from splenic adrenergic nerves, it is conceivable that they mediate phentolamine-induced vasospasm in the presence of functional sympathetic innervation.