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In vivo and electron microscopic studies of the splenic microvasculature in mice

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Key words. Spleen; mice; microvasculature, in vivo microscopy, electron microscopy; white pulp; red pulp.

The spleen of many adult mice is an erythropoietic organ. Since this organ is amenable to in vivo microscopic study, we have used the mouse spleen as a site to study the interrelationships between erythropoiesis and the microvascular compartment of the hemopoietic microenvironment[30–36,43–45].

However, neither the 'open' nor the 'closed' theory of splenic circulation by themselves adequately explained our observations of the microcirculation during conditions of erythropoietic stimulation and suppression in mice with normal and elevated hematocrits (polycythemia). As a result, it was necessary to elucidate the pathway of blood flow through the erythropoietic red pulp. This pathway has been the subject of recurring controversy[48,49] since most in vivo microscopic studies of the mammalian spleen and a few electron microscopic studies suggest that in the red pulp most of the blood flows within channels lined by endothelium[4,27,40–42]. However, other in vivo microscopic studies[18,29,39] and most transmission and scanning electron microscopic investigations[1–3,5–10,12,13,15,19,21–25,28,38,47,50–52] suggest that most of the circulation of blood within the red pulp is not contained by endothelium, but flows through a meshwork of reticular cell processes to reenter the vasculature by penetrating the endothelial wall of the venous sinuses.

In an attempt to resolve this controversy and provide the morphologic data necessary for improving the interpretation of the results of other experiments, the microvascular system of the mouse spleen has been studied using improved, high resolution in vivo microscopic methods[30–38,43,44] and the light microscopic images secured in vivo were correlated with the ultrastructure of the red pulp obtained by transmission electron microscopy.

The spleens of more than 1500 CF_1 and CD mice have been studied by in vivo microscopy[36,37]; 40 spleens were examined electron microscopically. To study the spleen in vivo, the animal first is anesthetized with either Urethane (2.5 mg/g i.p.) or sodium pentobarbital (0.03 mg/g i.p.). Then the tip of the organ is gently exteriorized through a 1-cm, left subcostal incision and positioned over a mica window in a specially designed microscope stage. The window overlies a long working distance condenser of a modified Leitz Panphot microscope. The spleen is covered by a piece of Saran (Dow Chemical) cemented to a movable 'U'-shaped frame. The Saran holds the organ in position and limits movements induced by respiration and the heart, yet is flexible enough to avoid compression of the underlying splenic microvasculature. Homeostasis is maintained by

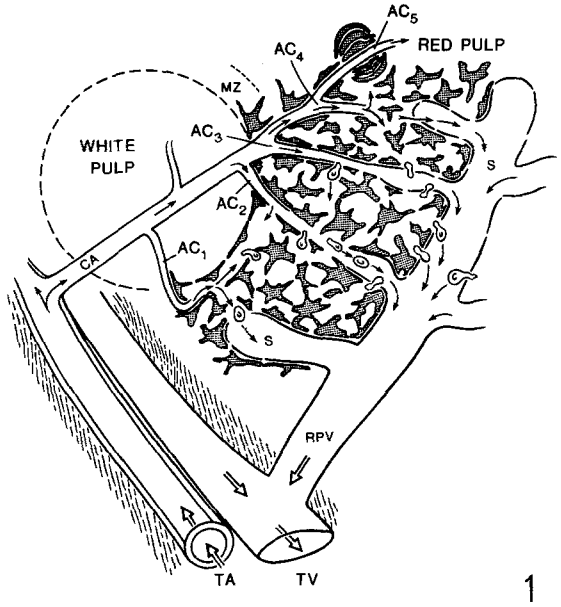
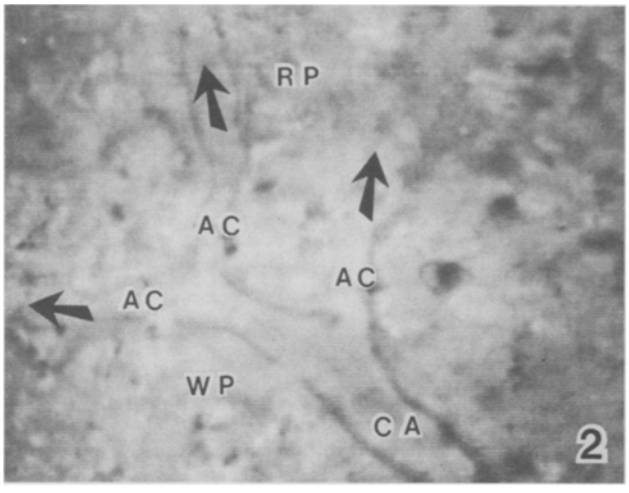
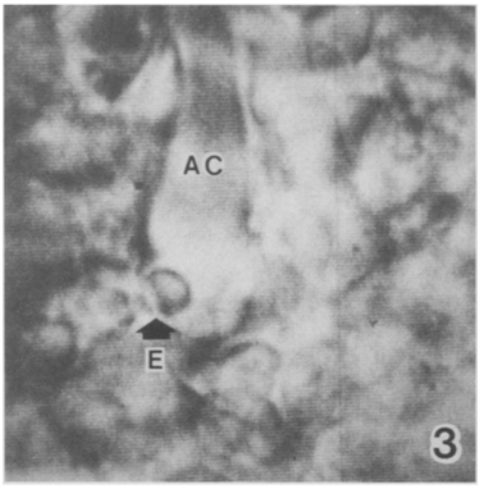
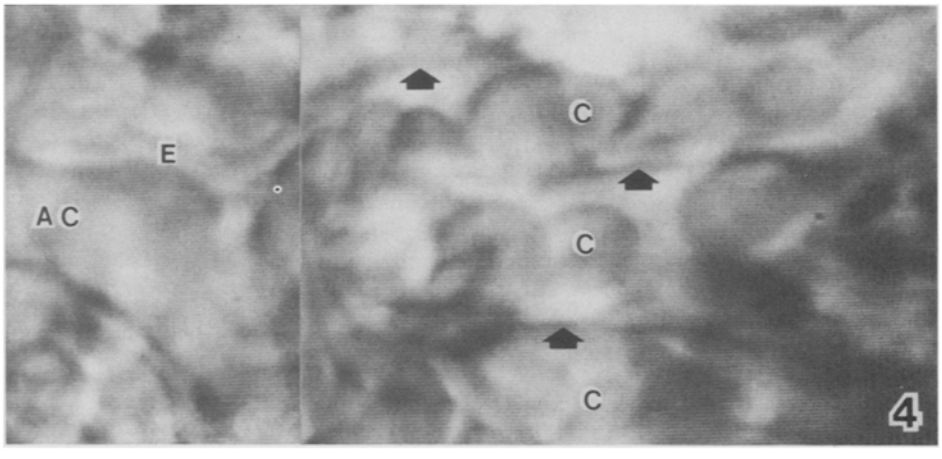
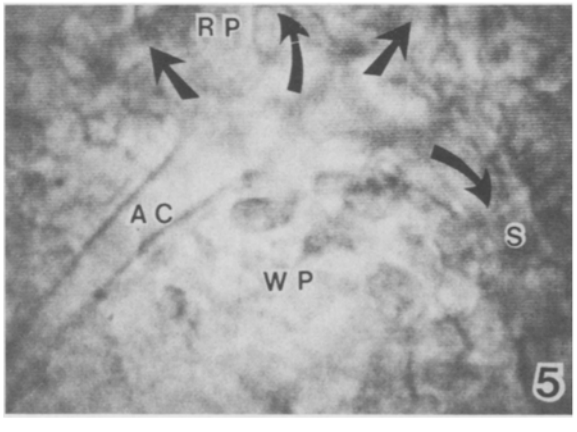


Figure 1. Diagrammatic illustration of the splenic microvasculature[38]. TA: trabecular artery; TV: trabecular vein; CA: central arteriole; AC_1 and AC_2: 'arterial' capillaries terminating in the marginal zone (MZ); AC_3 and AC_4: 'arterial' capillaries terminating in the red pulp, AC_5: sheathed 'arterial' capillary terminating in the red pulp (not developed in the mouse); S: venous sinus; RPV: red pulp venule. Arrows indicate direction of blood flow. Solid black lines indicate the endothelial lining of vessels.









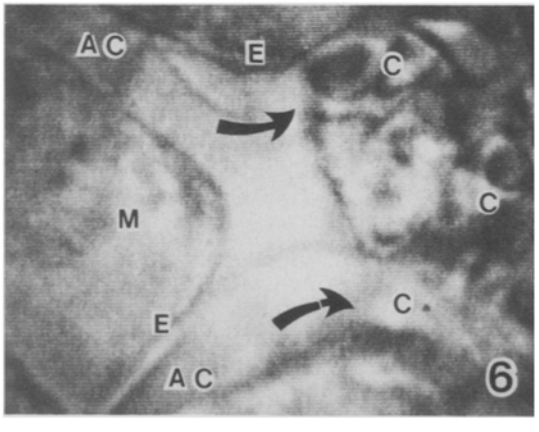
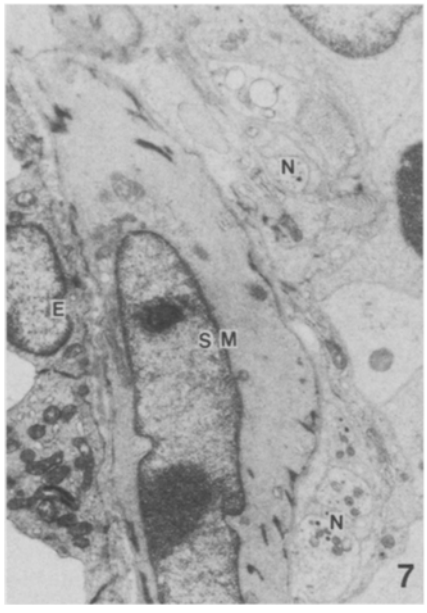
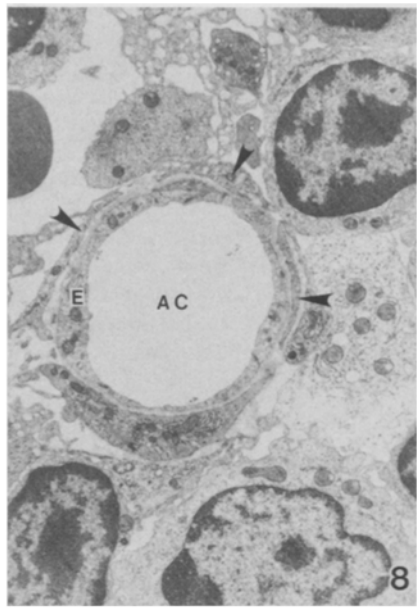


Figure 2. In vivo photomicrograph of central arteriole (CA) in white pulp (WP) branching into 'arterial' capillaries (AC) terminating in the red pulp (RP). Arrows indicate direction of flow. Figure 3. In vivo photomicrograph of 'arterial' capillary (AC) terminating in the red pulp. Note red blood cell penetrating aperture in endothelium (E). Figure 4. In vivo photomicrograph of 'arterial' capillary (AC) terminating in red pulp. Note vascular channels (C) in the red pulp formed by reticular cell processes (arrows) which are dimensionally similar to the endothelium (E) of the 'arterial' capillary. Figure 5. In vivo photomicrograph of 'arterial' capillary (AC). Arrows indicate direction of flow through apertures in endothelium and into a sinus (S). WP: white pulp; RP: red pulp. Figure 6. In vivo photomicrograph of two 'arterial' capillaries (AC) terminating in the red pulp adjacent to a megakaryocyte (M). C: channels in red pulp. Arrows indicate direction of flow. Note loss of endothelium at E.





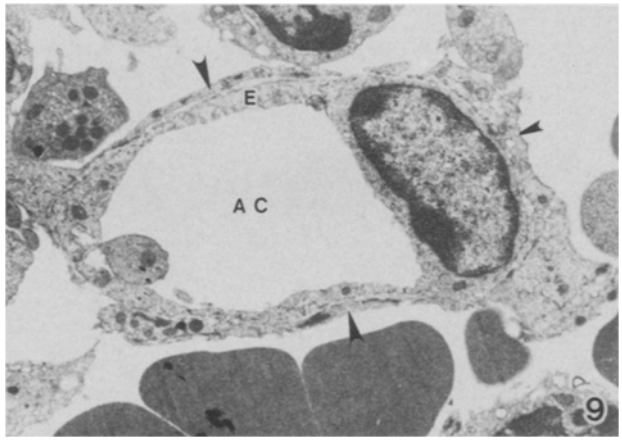
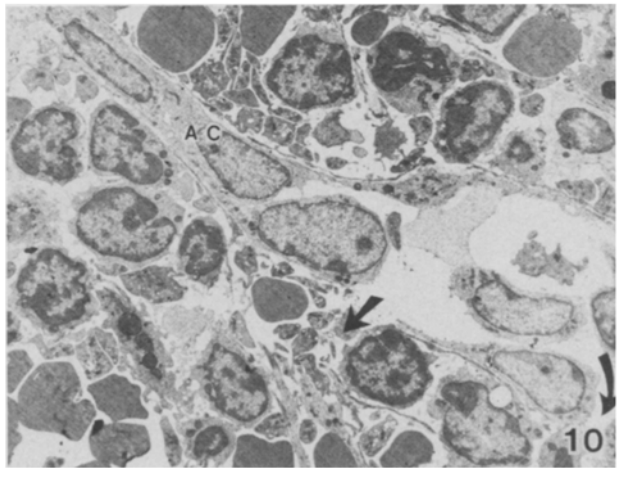


Figure 7. Central arteriole in white pulp. E: endothelium; SM: smooth muscle; N: nerves. × 9225. Figure 8. 'Arterial' capillary (AC) in marginal zone. Arrows indicate reticular cell processes separated from the endothelium (E) by basal lamina. × 4600. Figure 9. 'Arterial' capillary (AC) in red pulp. Arrows indicate reticular cell processes separated from the endothelium (E) by basal lamina. × 7275.



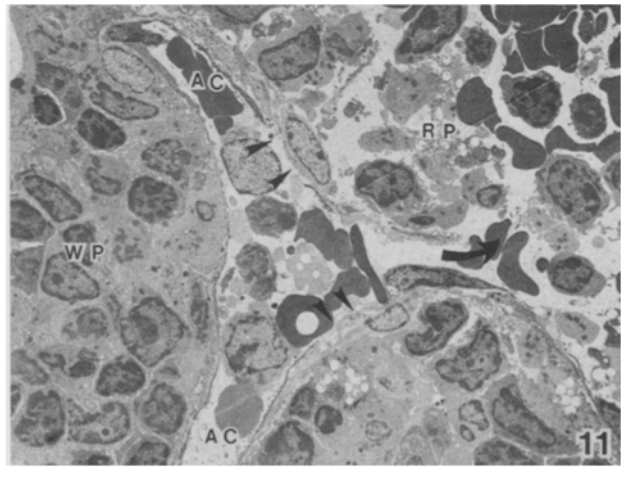


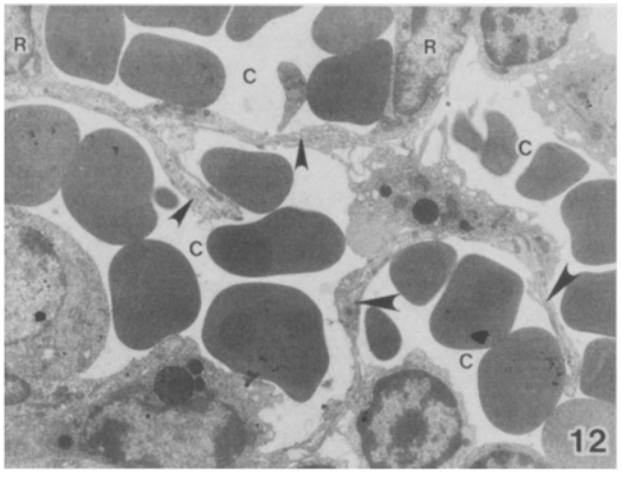
Figure 10. 'Arterial' capillary (AC) terminating in the red pulp. Note discontinuities (arrows) in the endothelium to permitting egress of blood into red pulp. × 2270. Compare with figure 3.

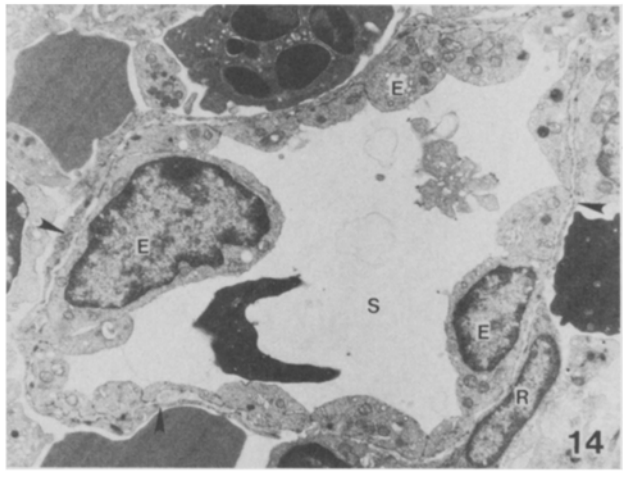
Figure 11. Two 'arterial' capillaries terminating in the red pulp (RP). Note breaks in the endothelial continuity (arrows). × 1332. Compare with figures 5 and 6.

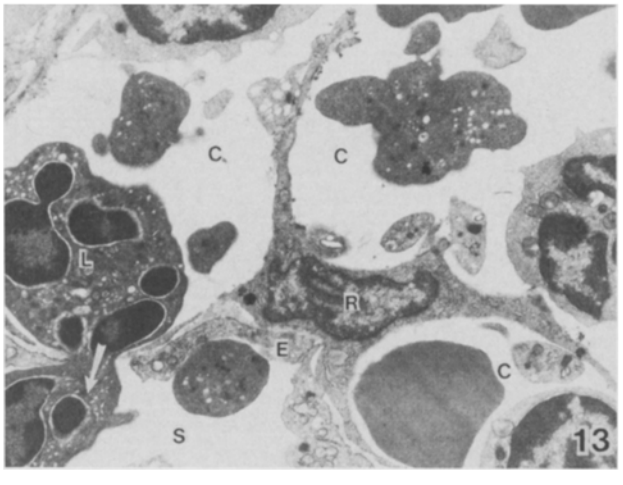
constant irrigation with Ringer's solution over the surface of the organ. The temperature of the Ringer's is maintained automatically at body temperature by electronically controlled, proportional regulating heaters. Using this system the surface of the organ is maintained at rectal temperature ± 0.3 °C.

The spleen is transilluminated with monochromatic light (550–750 nm) obtained from a Leitz prism monochrometer equipped with a Xenon lamp (XBO-150) through a long working distance Leitz condenser. The microscope tube is then positioned over the transilluminated area and microscopic images of the microvasculature secured at magnifications of 200–1350X using Leitz 22X, 50X, 55X, 75X, 80X, or 90X water immersion objectives with appropriate oculars. The resulting optical images are either studied directly or are projected onto the photocathode of a silicon vidicon television system (Cohu). Video images are either taped using either a Panasonic ½″ or Sony ¾″ U-matic video tape recorder or are kinérecorded at 30 fps from a 17″ video monitor using a 16 mm Arriflex 16-S motion picture camera equipped with a special motor to synchronize the framing of the video and photographic images. Kodak TRI-X reversal film is used.

The use of a monochrometer permits the selection of wavelengths of light which are selectively absorbed or transmitted by specific tissue and cellular components. When such differences of absorption are sensed by the television tube and converted into an electronic image, the contrast between tissue and cellular components can be enhanced further by adjustment of the brightness and contrast controls on the video monitor. Thus, the images of a particular structure can be enhanced or suppressed depending upon the wavelength of light selected and the settings of the television system. For studying the microvasculature of the spleen it is useful to transilluminate the organ at wavelengths of light between 575 and 750 nm to eliminate the absorption of light by the hemoglobin contained in the numerous erythrocytes flowing or sequestered in the red pulp. This not only increases the amount of light transmitted through the spleen but also enhances the definition of endothelium and other cellular components. When such images are televised using a silicon-vidicon having a peak spectral







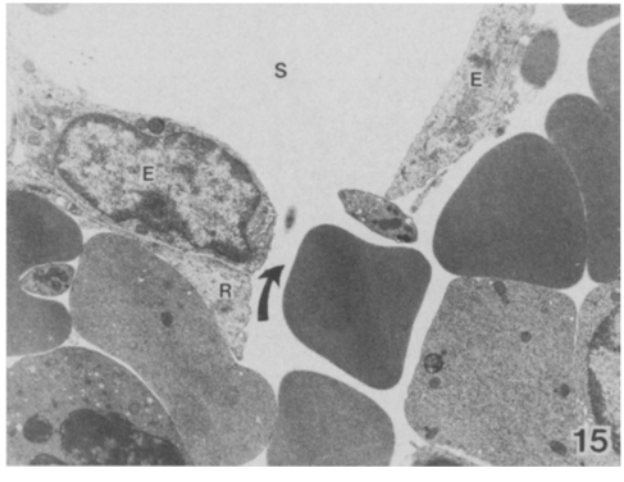


Figure 12. Vascular channels (C) in the red pulp formed by reticular cells (R) and their processes (arrows). × 3570. Figure 13. Vascular channels (C) in the red pulp formed b y reticular cells (R) and their processes. Note apposition of reticular cell processes to the wall of a venous sinus (S). A leukocyte (L) is diapedesing through an aperture in the sinus endothelium. × 6790.

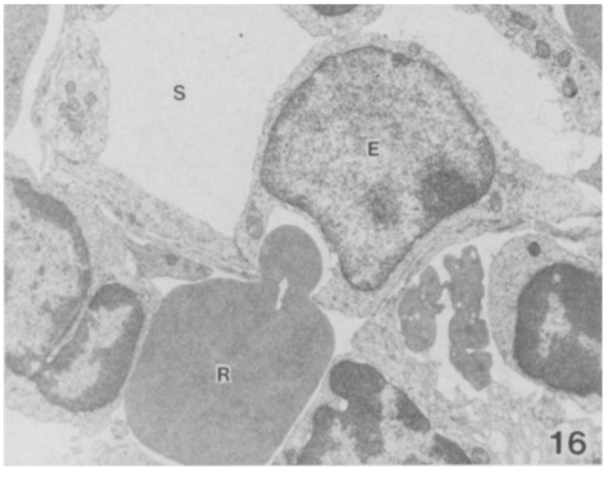
Figure 14. Venous sinus (S) in the red pulp. E: endothelium; R: reticular cells and processes (arrows). × 5380. Figure 15. Large aperture (arrow) in endothelium of venous sinus (S). E: endothelium; R: reticular cell. × 5840.

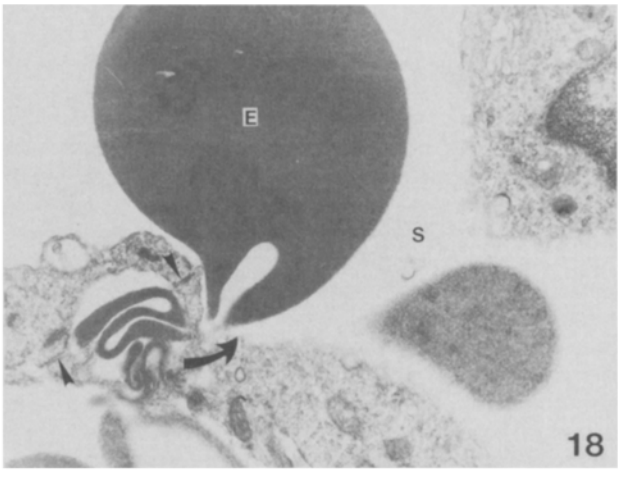
response between 600 and 800 nm, the following are observed in most spleens at very low light levels (10^{-2} fc): differentiation of the red and white pulp, differentiation of the microvasculature into arterioles, capillaries, sinuses and venules; patterns of blood flow through these vessels; the shape and deformation of individual blood cells; the endothelium of most vessels; stagnation of blood (storage) in the red pulp; and some cytoplasmic and nuclear detail. The measured resolution of this system under optimal conditions is 0.3–0.5 µm.

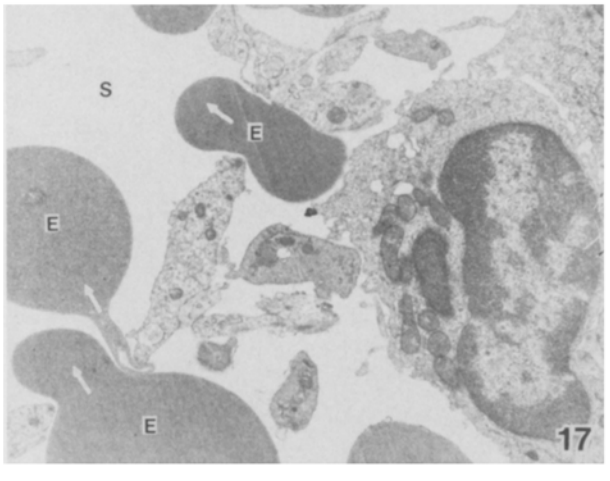
For the ultrastructural studies, the spleens of anesthetized animals are fixed by perfusion at physiological pressure using two separate aldehyde solutions at room temperature[26]. The first solution (washout) contains 0.5% paraformaldehyde, 0.1 gluteraldehyde, 0.05% $CaCl_2$, 0.025% $AlCl_2$ and 1% procaine hydrochloride (300 mOs). The second solution (fixative) contains 0.3% paraformaldehyde, 1.5% glutaraldehyde, 0.05% $CaCl_2$ and 0.025% $AlCl_2$ (400 mOs). The pH of both solutions is adjusted to 7.3 with 0.2 N sodium cacodylate buffer. After being perfused the spleens are removed and minced into 1–2 mm blocks. The tissue is post-fixed in 2% osmium in cacodylate buffer at room temperature for 1 h. Then, the tissue is dehydrated through graded ethanols, cleared in propylene oxide, and embedded in Spurr or Epon. Tissue sections are cut with glass or diamond knives on a Porter-Blum ultramicrotome (Sorval MT-2). Semithin, 1-µm sections are stained with 1% toluidine blue in 1% borax solution. Thin sections are stained with 4% uranyl acetate followed by 1% citrate and examined using a Phillips 301 or JEOL 100 CX transmission electron microscope.

In vivo light microscopic and electron microscopic study of the murine spleen has resulted in the concept of the splenic microvasculature presented diagrammatically in figure 1.

In vivo the white pulp is distinguished easily by the presence of the central arteriole surrounded by closely packed mononuclear cells (lymphocytes) of fairly uniform size (figs 2 and 5). This area is relatively avascular and only an occasional capillary is seen in the white pulp both in vivo and electron microscopically. The few capillaries seen in the white pulp originate from the cen-







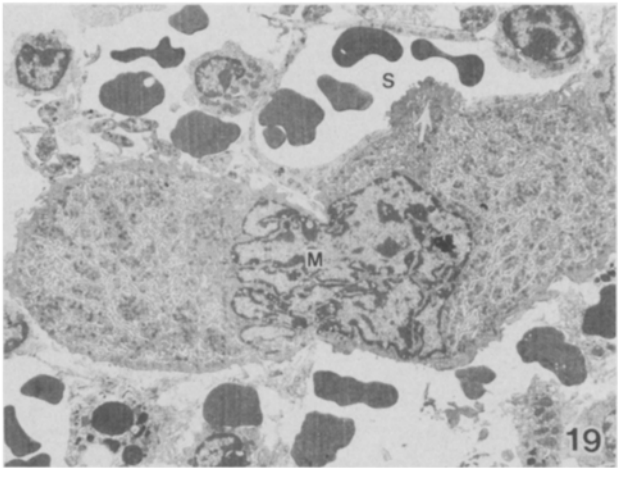


Figure 16. Reticulocyte (R) penetrating an endothelial cells (E) of venous sinus (S). × 7275. Figure 17. Erythrocytes (E) penetrating endothelial wall of a venous sinus (S). Note deformation of cells during passage through the apertures. Compare with figures 20–22. × 7785.

Figure 18. Erythrocyte (E) in passage (arrow) through interendothelial cell aperture into venous sinus (S). Arrowheads indicate junctional complexes between contiguous endothelial cells. × 15,370. Figure 19. Process (arrow) of a megakaryocyte (M) protruding through aperture in the wall of a venous sinus (S). × 1950.

tral arterioles and after coursing through the white pulp terminate in the marginal sinus or in vascular channels in the red pulp. Ultrastructurally, these capillaries consist of an continous endothelium surrounded by a basal lamina. The central arterioles also are lined with a endothelium having no discontinuities and have a continuous basal lamina. The latter are surrounded by a single, or at the most, a double layer of smooth muscle cells (fig. 7). During its course through the white pulp the central arteriole gradually loses its envelopment of smooth muscle cells so that upon entering the red pulp, this vessel has the appearance of a capillary composed of a continuous endothelium surrounded by a complete basal lamina plus an investment of cytoplasmic processes of reticular cells (figs 8 and 9). The endothelium of these vessels often protrudes into the lumen, contains microfilaments suggestive of contractile activity, is very responsive to vasoactive substances and nerve stimu-

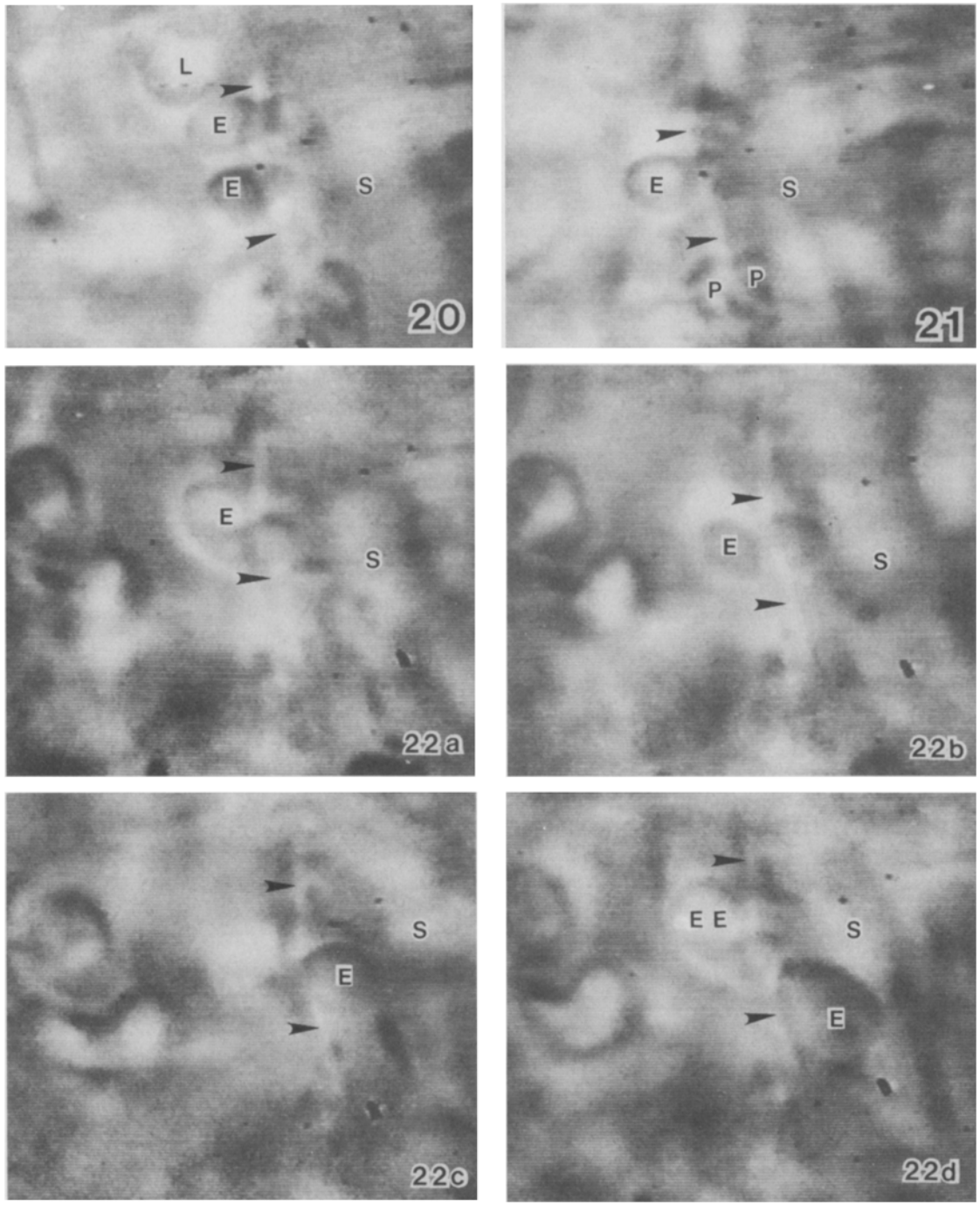
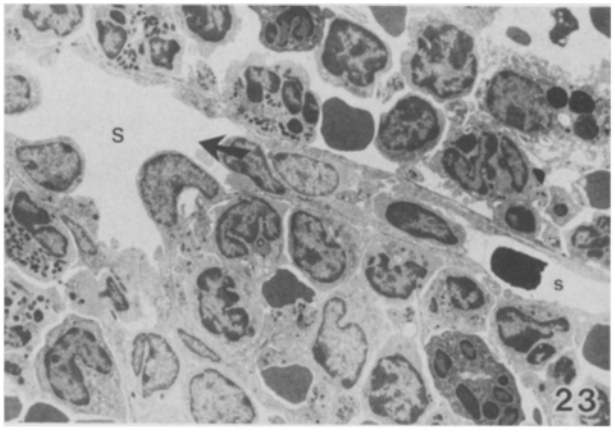
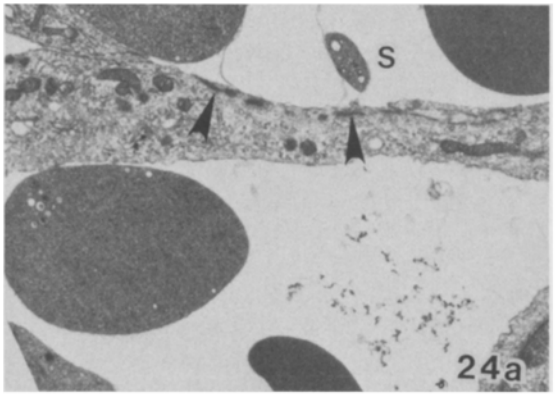


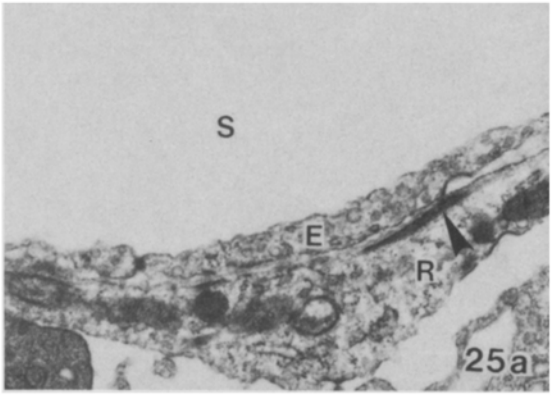
Figure 20. In vivo photomicrograph of erythrocytes (E) being deformed in passage through endothelial wall (arrows) into a venous sinus (S). L: leukocyte. Figure 21. In vivo photomicrograph of an erythrocyte (E) beginning to penetrate endothelial wall (arrows) of a venous sinus (S). Note platelets (P) attached to endothelium. Figure 22. In vivo photomicrographs of an erythrocyte (E) being deformed during passage through the endothelial wall (arrows) into a venous sinus (S). Video frames a, b, c and d are each separated by 0.2 sec. Note in figure 22d that a second erythrocyte (EE) already has begun to penetrate the same aperture just vacated by the original erythrocyte (E).

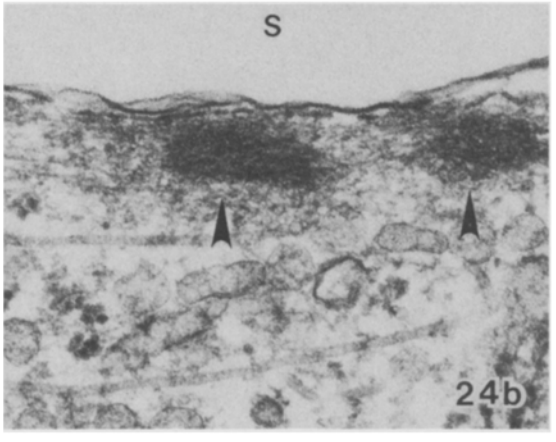
lation; and regulates flow into the red pulp[30–35, 43, 44]. While numerous nerve fibers can be found in the thin adventitia of central arterioles (fig. 7), no nerves have been observed to be associated with the 'arterial' capillaries (figs 8 and 9)[45, 46].

Near the terminations of 'arterial' capillaries in the marginal zone and red pulp, discontinuities in the wall are noted in vivo (fig. 3) and electron microscopically (fig. 10). Erythrocytes flow laterally through these discontinuities from the 'arterial' capillary into the red pulp (fig. 3). Most of the blood, however, flows from the endothelially-lined 'arterial' capillary into either the marginal sinus or into channels within the red pulp formed solely by the cytoplasmic processes of reticular cells (figs 4–6). As a result, blood flowing through two adjacent channels frequently is separated by only a single cytoplasmic process which is dimensionally similar to endothelium (figs 4, 12, 13). Where 'arterial' capillaries empty into the red pulp, the processes of the reticular cells are in close apposition to the endothelium of the 'arterial' capillaries (figs 10 and 11). In vivo this relationship frequently creates the appearance of a channel completely lined by endothelium (fig. 4) as was mistakenly interpreted in earlier in vivo microscopic studies using lower magnification[27, 40]. This is especially true when the 'arterial' capillary terminates in close proximity to a sinus and the continuing channel provides a direct pathway for blood flow into the sinus









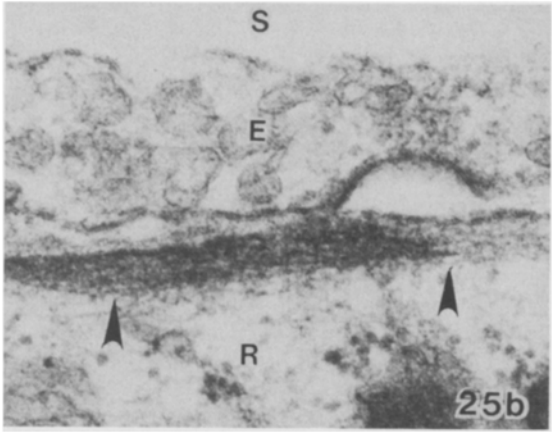


Figure 23. Small sinus (s) joining a larger, more developed sinus (S). × 1950. Figure 24. Microfilaments (arrows) in endothelial cell of venous sinus (S). a: × 6750; b: × 67,650. Figure 25. Microafilaments (arrows) in reticular wall process (R) adjacent to endothelium (E) of a venous sinus (S). a: × 6750; b: × 71,000.

(figs 1 and 5). A few such 'arterial' capillaries appear to connect directly with sinuses, but generally the endothelial continuity of such connections is not complete (fig. 5). These direct channels appear to function as arteriovenous (AV) shunts and may be the morphologic equivalent to 'fast' circulatory compartment predicted by erythrocyte washout studies[14,20].

The patterns of blood flow in the red pulp are complex and vary from moment to moment. For example, blood cells flow rapidly through the channels in the red pulp with minimal or no distortion and a few seconds later the flow is be reduced to only a few erythrocytes being highly distorted as they slowly pursue a tortuous pathway through the red pulp having many of the channels congested with blood cells. During such periods of congestion, flow always continues in the direct pathways described above with the result that the majority of blood flow bypasses the complex channels in the red pulp to enter the splenic sinuses. When the red pulp is not congested, the majority of the blood leaves and red pulp by passing through apertures in the endothelium of sinuses (figs 13, 15–22). Most of these apertures are of such size that blood cells are deformed during their passage from the cords into the sinus (figs 13, 16–18, 20–22). Platelets transiently adhere to and plug the apertures (fig. 21) thereby momentarily limiting the entry of erythrocytes into the sinuses. The transendothelial transit of leukocytes is even slower and appears to be accomplished by diapedesis (fig. 13). Some apertures, however, are large enough to allow passage of blood cells without them being deformed in passage (fig. 15). These frequently are associated with the 'functional' AV shunts described above. The apertures are formed as gaps between contiguous endothelial cells at sites where the endothelium is not effaced on its abluminal surface by a basal lamina or by adventitial processes of reticular cells (figs 13, 15–17). The sinuses in mice are not extensive (figs 14 and 23) and have a relatively attenuated endothelial lining when compared to those of the rat; both the endothelial cells and the reticular cells that efface their abluminal surfaces contain numerous microfilaments (figs 24 and 25) suggestive of a contractile function as has been reported in other species[13,16]. The latter may represent the structural mechanism for altering the apertures and regulating cellular egress[16,35,36]. Of particular interest is the observation of what appeared to be newly formed erythrocytes penetrating the cytoplasm of the endothelial cells (fig. 16). Such penetration also has been described in the bone marrow[11,17].

A limited number of in vivo microscopic studies of the rat spleen suggest similar microcirculatory patterns except that the sinuses are more highly developed.

In summary, the use of improved in vivo microscopic methods has permitted a reevaluation of the 'open' vs 'closed' theory of blood flow through the murine splenic red pulp. The results strongly suggest that, in general, there is little or no continuity of endothelium through the splenic red pulp. Instead, blood flows from 'arterial' capillaries lined with endothelium into channels within the red pulp formed by the cytoplasmic processes of reticular cells. In vivo these processes appear similar to endothelium leading earlier investigators to the conclusion of a 'closed' circulation. Blood leaves the red pulp by passing through apertures in the endothelium of the sinuses. Where 'arterial' capillaries terminate near large apertures in the sinus wall, a 'functional' arteriovenous shunt may be formed allowing blood to bypass the more tortuous channels in the red pulp, especially during periods of congestion in the red pulp.

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Innervation and vascular pharmacodynamics of the mammalian spleen

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Key words. Spleen, mammalian; innervation; vascular pharmacodynamics.

1. Introduction

The principal goal of the following treatise is to consider the effect(s) of potentially vasoactive substances and synthetic agonists, antagonists, or other inhibitors, on the capsule and vasculature of the mammalian spleen. The anatomic distribution of autonomic and sensory nerves as well as the role of neural mechanisms in splenic function also are evaluated in a number of species. Where relevant, an assessment is made of the pathophysiologic significance of elevated levels of neurotransmitters, or other humoral agents, on 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 low-flow states. Lastly, the limitations of the experimental per-