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Capillary Leakage in Inflammation

A Study by Vascular Labeling

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The local injection of pure inflammatory mediators induces venular leakage. To test the effect of endogenous mediators from dying tissue on vascular leakage, the authors devised an experimental model simulating an infarct, whereby living vessels would be exposed to fragments of organs undergoing aseptic necrosis. Tissues from donor rats were implanted aseptically in the cremasteric sac. Control rats were implanted with materials deemed to be as close as possible to nonirritating: boiled tissues and spheres of Teflon or glass. At different points the rats were injected intravenously with carbon black and killed an hour later. Whole cremaster mounts showed that vascular labeling was strictly venular up to 8 hours, mixed with capillary labeling between 12 and 24 hours, and mainly or exclusively capillary at 48 hours. Histology showed an acute inflammatory infiltrate in the labeled areas. A similar but weaker labeling pattern accompanied by milder inflammation was seen in controls. These results indicate that the vascular leakage in aseptic inflammation is biphasic, first venular, then capillary; and that the capillary phase is induced by the inflammatory reaction itself, possibly through a form of diffuse angiogenesis. (Am J Pathol 1990, 137:1353–1363)

The notion of vascular leakage in inflammation dates from 1873, when Julius Cohnheim realized that the inflammatory tumor was best explained by an increased permeability of the vascular wall.[1] Limited by the resolving power of the light microscope, Cohnheim could not further define which segment of the microcirculation had become leaky, nor was the question relevant in his day; it was generally assumed that the change concerned the finest vessels, and the capillary leakage of inflammation remained dogma for nearly a century.

In 1961, thanks to electron microscopy and to the availability of chemically pure inflammatory mediators, it became apparent that histamine, serotonin, and bradykinin induced gaps in the endothelium of the venules, whereas the capillaries were almost completely spared.[2] It also was found that colloidal pigments injected intravenously became trapped in the walls of the leaky microvessels, forming deposits large enough to be recognized by light microscopy; this observation became the basis of a method, known as *vascular labeling*, that made it possible to define with considerable precision any leaky segment in the microvascular network.[3]

Studies based on the principle of vascular labeling then showed that local injury (mechanical, thermal, or toxic) caused leakage to occur indiscriminately—as expected—from all segments of the microcirculation: arterioles, capillaries, and venules; this was defined as 'leakage by direct injury.'[4–7] By contrast, as new vasoactive mediators were discovered and tested, it was found that they consistently induced a 'histamine-type' vascular leakage—from the venules only.[8,9] In the case of histamine, the target selectivity could be explained by the predominance of receptors on the venular wall[10]; and a cellular mechanism for the response was found, namely the contraction of endothelial cells, again limited to the venules.[11] Thus it seemed reasonable to conclude that any venular leakage in inflammation would be due to mediators, and any capillary leakage to direct injury.

Although the studies mentioned above—performed with pure mediators—are obviously pertinent, it is equally obvious that they do not entirely duplicate the conditions that prevail in a natural setting of inflammation. Therefore we devised a model in which living vessels would be exposed not to a purified chemical agent, but to a mixture of endogenous mediators, as would be produced during the natural course of a local injury. We excluded infection, because it implies a double mixture of mediators, one group arising from damaged tissues, the other from the

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bacteria themselves (including toxins and enzymes capable of causing direct injury). Our choice fell on aseptic necrosis on the assumption that dying and necrotic tissue would become a reproducible source of inflammatory mediators. For reasons that will become apparent in the Discussion, we did not use a typical infarct, but a model in which a mass of dying or dead tissue is placed next to an intact vascular network acting as the target tissue. The result came as a surprise: vascular labeling showed that a phase of venular leakage blends into a phase of intense capillary leakage.

Materials and Methods

Preliminary experiments showed that the most convenient way to expose normal vessels to dying tissue was to take, under aseptic conditions, a sample of tissue (usually liver or kidney) from one donor rat, and to implant it into a recipient rat, in the peritoneal pocket represented by the cremasteric sac (Figure 1). The vessels of the cremaster muscle then could be labeled at a chosen time by an intravenous injection of carbon black, then fixed and studied by transillumination as described previously.[3]

Animals

We used 122 Wistar rats (Harlan Sprague Dawley, Inc., Indianapolis, IN). We chose large rats (400 to 600 g) for donors so that the size of the implants would approximate the size of infarcts as seen, for example, in human kidneys. As to the recipients, best were rats in the 400- to 500-g range; those under 300 g were more prone to retract the cremaster and thus extrude the implant into the abdominal cavity. All animals were maintained and tested in accordance with recommendations in the *Guide for the Care and Use of Laboratory Animals*[12] and the guidelines of the Animal Care Advisory Committee of the University of Massachusetts Medical School.

Harvesting of the Implants

A donor rat was anesthetized with ether, then injected intravenously with 0.1 ml of penicillin G solution (100,000 U/ml, Eli Lilly and Co. Indianapolis, IN) and immediately thereafter with a 0.1-ml of a solution of streptomycin sulfate (200 mg/ml, Eli Lilly and Co., Indianapolis, IN); the rat died within 5 to 15 seconds. We assumed at this point that both the liver and the kidneys contained blood saturated with antibiotics. The abdomen then was opened aseptically; the kidneys and liver, and when necessary also the heart, abdominal muscle, testis, and epididymal fat were removed to sterile petri dishes. The kidneys were freed of adipose tissue by decapsulation with sterile forceps and weighed 1.3 to 2 g. From the left and median lobes of the liver four samples were excised, measuring approximately 22 × 12 × 6 mm and weighing 1.1 to 2.0 g, and therefore approximating the size and shape of a rat kidney. Implants were weighed aseptically.

Surgical Procedures

Implantation was performed under aseptic precautions. The rat abdominal wall was shaved and disinfected with Betadine (Purdue Frederick, Norwalk, CT). A 30-mm midline laparotomy incision was made, and the right testis was gently eased into the peritoneal cavity by manipulation through the scrotal skin. The selected implant was seized with a toothless hemostatic forceps, introduced through the inguinal canal into the cremasteric sac, and deposited there (Figure 1); the same operation was repeated on the left side. The abdominal wall was closed with separate stitches and the skin with surgical clips; the rat then was given a single dose of subcutaneous antibiotics (0.1 ml each of a penicillin G 100,000 U/ml and streptomycin sulfate 200 mg/ml).

Control Experiments

Implants

Boiled tissues: Fragments of liver and whole kidneys were boiled in distilled water for about 10 minutes, allowed to cool in a sterile petri dish, and implanted as usual. Teflon spheres (#BT-8, 1/2-inch in diameter, 2.28 g) were obtained from Small Parts Inc. (Miami, FL). Hollow glass spheres (with glossy or frosted surface, diameter 12 mm, weight 1 g) were especially blown for us by Radnoti Glass Technology, Inc. (Monrovia, CA).

Sham Operation

A tissue implant was introduced into the cremasteric sac as described, but retrieved immediately thereafter.

Normal Controls

Two rats that received no carbon were killed and their cremasters prepared and fixed for electron microscopy.

Histamine Controls

Normal rats were injected subcutaneously over the right cremaster with 0.1 ml histamine phosphate (histamine

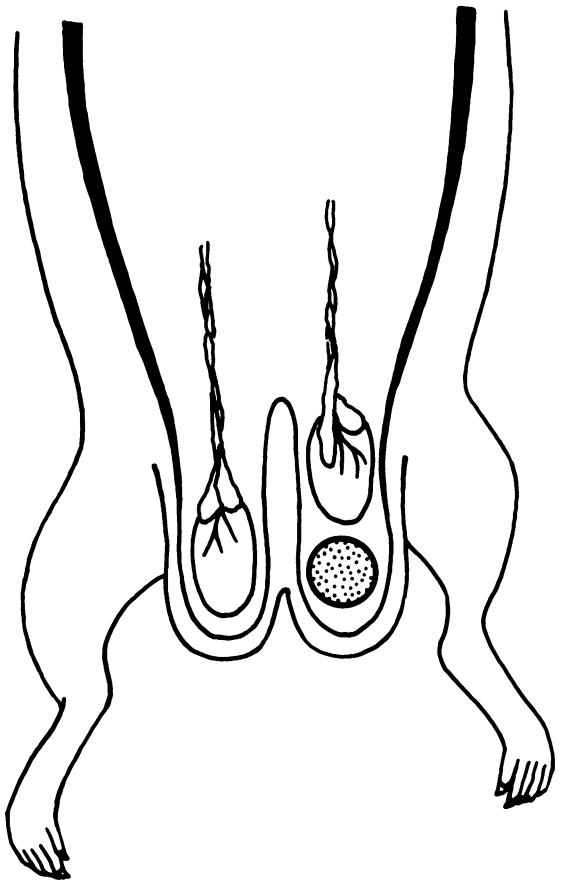

Figure 1. *Scheme of the cremasteric sacs in a recipient rat with an implant in place (stippled circle).*

0.1 mg/ml, Eli Lilly and Co.) and intravenously immediately thereafter with carbon black.

Implanted Rats: Vascular Labeling of the Cremasters

At the selected time after implantation, the rat was anesthetized with ether, and injected intravenously with 0.1 ml per 100 g body weight of a suspension of carbon black in an aqueous solution of hydrolized gelatin (Carbon Black Dispersion #8, Faber-Castell Corp., Newark, NJ), to a maximum dose of 0.3 ml. An hour later, the animal was killed while under heavy ether anesthesia by making an incision in the chest and clamping the heart. The cremasters then were excised.

Overall Experimental Plan

All animals were killed 1 to 2 hours after the intravenous injection of carbon, thus allowing ample time for labeling of the leaky vessels and for clearance of the remaining carbon. The experimental animals were killed in groups of 7 to 14 and the control and sham rats in groups of 1 to 5.

Preparation of the Cremasters for Light Microscopy

The cremasteric sacs were dissected out, opened, pinned on dental wax, and fixed in 10% neutral buffered formalin (Surgipath, Medical Industries Inc., Graysville, IL). After 24 hours or longer, representative strips (3 × 12 mm) were excised from labeled as well as from unlabeled areas, embedded in methacrylate, and 2-μ sections cut perpendicular to the long edge were stained with hematoxylin and eosin (H&E). The remaining cremaster muscle was stripped of excess subcutaneous connective tissue, cleared in two 24-hour changes of pure glycerol, and mounted flat for transillumination in glycerin jelly (10 g gelatin dissolved in 60 ml of distilled water and 70 ml glycerin with 1 ml of 90% phenol).

Electron Microscopy

The rats were killed as described above, the scrotal skin was excised, and the cremaster muscle was immediately flooded with 3% glutaraldehyde in 0.1 mol/l (molar) cacodylate buffer, dripped on as well as injected into the cremasteric sac; it then was pinned out, and 1-mm strips were cut perpendicular to the prevalent direction of the capillaries. After postfixation in 1.3% OsO_4 in 0.1 mol/l cacodylate buffer, the samples were processed through graded ethanols and propylene oxide, and embedded in epoxy resin (Epon-812; E. Fullam, Inc., Latham, NY) in such a way that most vessels would be cut transversely. Ultrathin sections were cut with an LKB Ultrotome III equipped with a Diatome diamond knife (Diatome-US, Fort Washington, PA), mounted on copper grids, stained with uranyl acetate and lead citrate, and examined with a Philips 301 electron microscope.

Bacterial Cultures

Selected implants (n = 14) were recovered under sterile conditions at 2 hours, 18 hours, 24 hours, 48 hours, and 3 days, and tested by a quantitative culture technique as follows.[13] A portion of the retrieved implant weighing approximately 5.0 g was resected from each specimen, weighed, and homogenized in a volume of sterile tryptic soy broth (TSB) equivalent to the weight of the sample. From the homogenate, 0.1 ml were transferred onto the

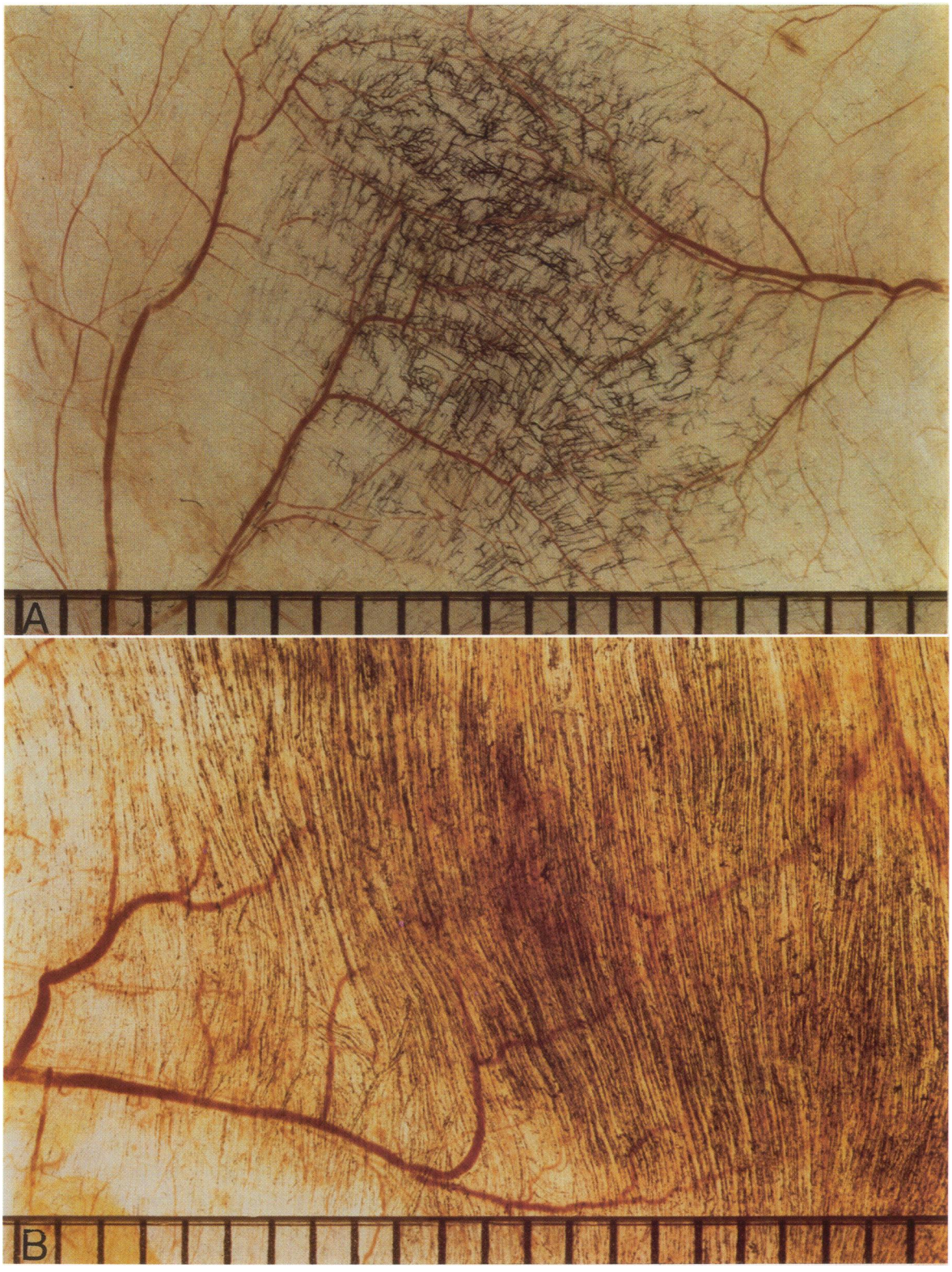
A
B

Figure 2. *Vascular labeling in the cremaster muscle (whole-mount preparations; scale in millimeters).* **A:** *Venular labeling induced by histamine. Note the typical branching pattern of the venules.* **B:** *Capillary labeling 48 hours after a kidney implant: the capillaries, arranged along the muscle fibers, appear as parallel arrays of labeled microvessels.*

following plate media: 5% sheep blood agar (SBA), enriched chocolate agar (choc), MacConkey agar (Mac) and Brucella-based anaerobic blood agar (BMB), thus achieving a final specimen dilution of 1:20. A 0.2-ml aliquot of the initial homogenate was also transferred into 10 ml of TSB, mixed, and then subcultured in 0.1 volumes to the same four plate media described previously (final specimen dilution of 1:1000). Last, 2.0 ml of the initial homogenate was inoculated into a tube containing 10 ml of sterile thioglycollate broth (Thio). The SBA, choc, and Mac plates and the Thio broth were incubated at 35 C in 5% to 7% CO_2 for 5 days and examined daily for evidence of growth. The BMB plates were incubated at 35 C in an anaerobic atmosphere for 7 days and examined on days 2, 4, and 7. When bacterial growth was noted on any media, colony counts were performed and organisms identified according to conventional criteria.[14]

Grading of the Vascular Labeling

The mounted cremasters were examined blindly by two observers and graded independently in a semiquantitative fashion. Two separate grades were assigned to each preparation: 1) proportion of venular *versus* capillary labeling, estimated by scanning the entire labeled area and expressed as V/C (eg, 7/3 would refer to 70% of the labeling in venules, 30% in capillaries); 2) overall intensity of the labeling, estimated with the naked eye (and at 10× when necessary) and graded from 0 to 5 (0 = no labeling, 1 = microscopic traces, 2 = a few labeled vessels barely visible by the naked eye, 3 = a distinct small patch of labeling, 4 = a large patch of labeling, 5 = extreme labeling throughout most of the cremaster).

Results

Bacteriologic studies showed that all the implants were sterile except for two (one each at 18 and 48 hours) that showed a few colonies clearly corresponding to skin contaminants (1 + *Staphylococcus* not *aureus* and *Proteus mirabilis*), presumably gathered during the sampling.

Gross Observations

Most of the implants remained in the scrotal cavity as expected; about 17% were lost, because cremasteric contraction had expelled them into the peritoneum. The implants became paler with time[15] and mild fibrinous adhesions were apparent at 48 hours. Surprisingly, adhesions with the testis never formed; however adhesions did form with the epididymis or with the mesotestis. Fluid exudate in the cremasteric sac was minimal but the cremaster itself was visibly edematous at 24 hours, and from then on the edema involved also the subcutaneous tissue over the cremaster.

Vascular Labeling

The pattern and degree of labeling at 24 hours were practically identical with the liver and kidney implants; at earlier stages (8, 12, and 18 hours) there was a slight tendency for kidneys to produce milder lesions, but at 48 hours the labeling was most pronounced with the kidney implants. Whenever vascular labeling was observed in the cremaster, it always developed in areas that were in contact with the implant. No labeling was ever observed in the testis, whereas it did develop in the epididymis and in the mesotestis. Two main patterns of vascular labeling were found: venular and capillary labeling. Labeled venules were recognizable by their typical branching pattern, similar to that produced by histamine (Figure 2A); this pattern is entirely different from that corresponding to labeled capillaries, ie, long, straight parallel lines (Figure 2B), because capillaries are aligned along the muscular fibers. Cremasters up to the stages of 4 hours showed relatively mild but 'pure' venular labeling (intensity 1 to 3, V/C: 10/0) (Figure 3A). At 8 hours, the number of labeled venules began to decrease; capillary labeling was first apparent at 8 to 12 hours, either mixed with labeled venules or in separate areas (Figure 3B). By 48 hours the labeling was almost strictly capillary (V/C: 3/7 to 0/10). The deposition of carbon in any individual capillary never reached the intensity observed in the venules, but it tended to be more uniformly spread along the length of the vessel (Figures 2B, 3C). Quantitative results are shown in Figure 4. At 3 days both labeling patterns—venular and capillary—suddenly became much less distinct and were replaced by scattered, punctate deposits (Figure 5), concomitant with the appearance of tight adhesions between the implant and the cremaster.

Foci of arteriolar labeling, of the type previously described as focal arteriolar insudation,[16] were noticed in 15% of the cremaster muscles at 8 hours, in 30% at 12 and 18 hours, 7% at 24 hours, and in 12% at 48 hours.

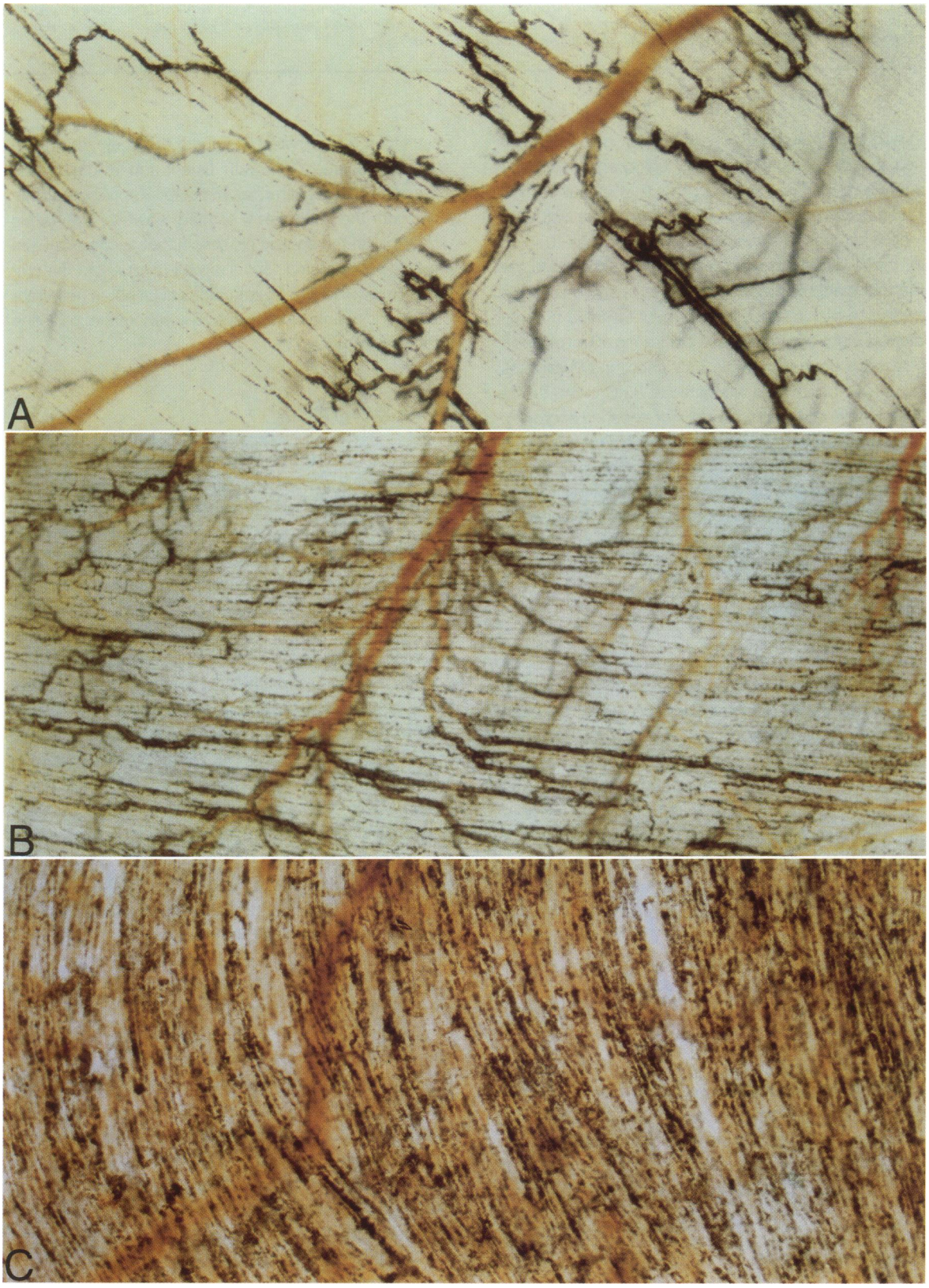
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Figure 3. *Vascular labeling of the cremaster muscle (whole-mount preparations: 25×).* **A:** *Mild venular labeling after exposure to a liver implant for 12 hours.* **B:** *Mixed venular and capillary labeling after exposure to a liver implant for 18 hours.* **C:** *Capillary labeling after 48 hours of exposure to a kidney implant.*

The intensity of vascular labeling with liver and kidney implants are similar except that maximal intensity is reached at 8 hours with the liver and somewhat later with kidney implants. As regards the proportion of labeled venules *versus* capillaries with implants of tissues other than liver and kidney, the results were qualitatively similar. In control experiments, at 48 hours boiled tissues, teflon, and smooth and frosted glass beads all produced a patch of mild, mixed labeling with capillary predominance (V/C for boiled tissues: 2/8 to 1/9; for teflon and glass: 5/5 to 1/9) (Figure 4). Sham operations produced minimal venular labeling at 2 hours and none at all at 48 hours.

Histology

The salient finding in cremasters exposed to implants of fresh tissue was an acute inflammatory infiltrate, heavier on the peritoneal side; it began to appear after 2 hours and increased thereafter, together with edema (Figure 6A–D). It was overwhelmingly composed of neutrophils up to 8 hours; between 12 and 18 hours, the mononuclear cells increased in number, so that by 24 to 48 hours they could be estimated as representing 85% to 95% of the inflammatory cell population. Some endothelial mitoses were found in the labeled areas. The same features, only milder, were noticed in all controls at 48 hours. Labeled vessels (especially capillaries) were not always easy to identify because of the thinness of the plastic sections (Figure 6D): a scattering of black grains along a vessel may be very obvious in a transilluminated whole mount, but in a 2-μ cross-section only a grain or two—or none—may be present. Damaged muscle fibers were found as early as 12 hours, in the inflamed area; they showed swelling, vacuolation, or coagulation necrosis, followed by macrophage invasion.

Electron Microscopy

Representative samples showed that both venules and capillaries were labeled with carbon black trapped against

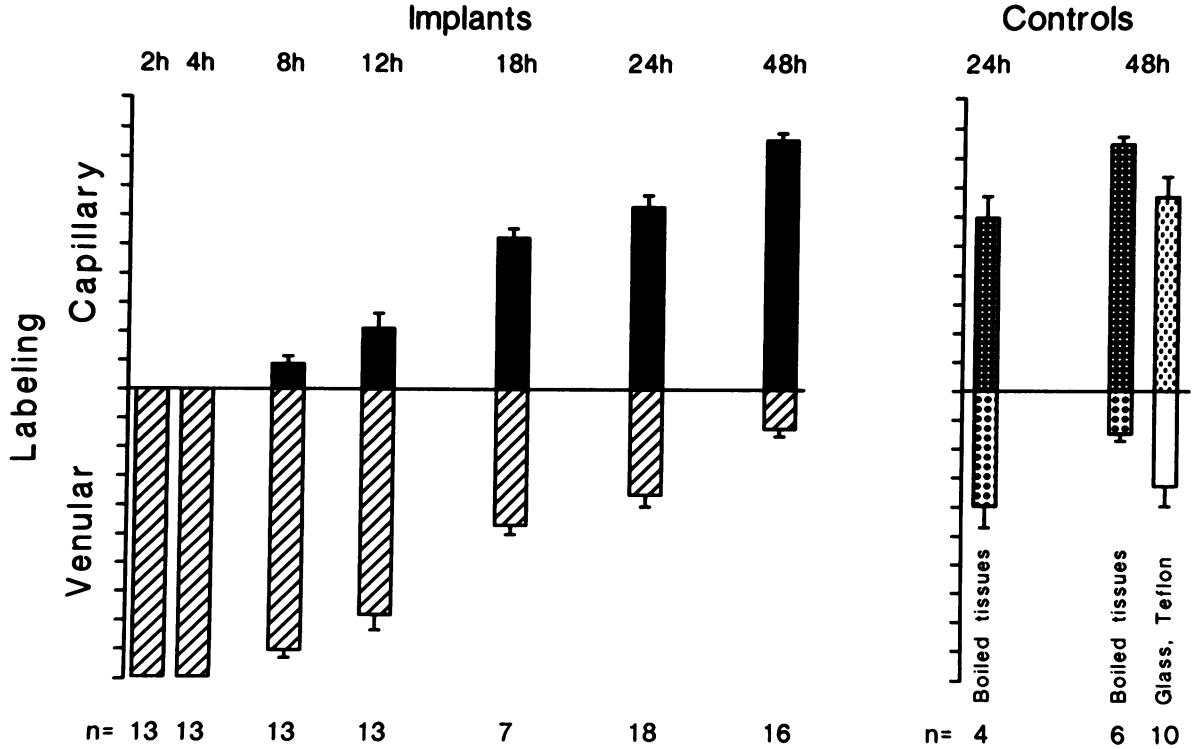

Figure 4. *Left: diagram showing the progressive shift from venular to capillary labeling (± SEM) in cremasters studied at different times, after implantation of liver or kidney. Right: control implants. (n = number of cremasters studied).*

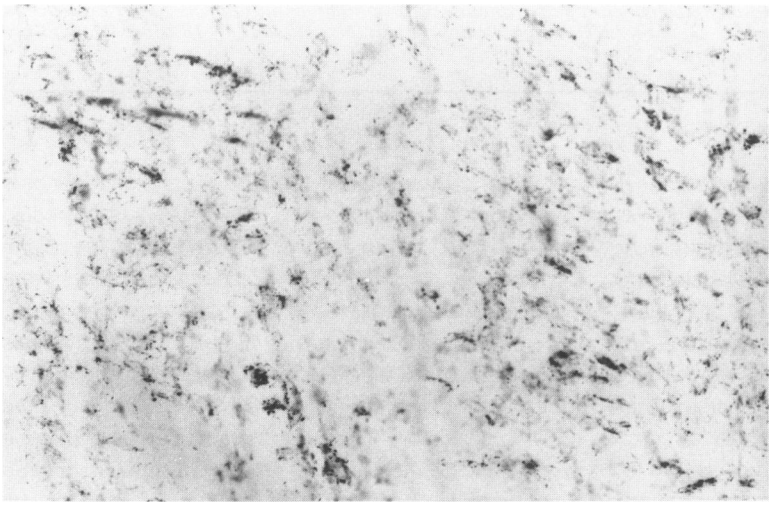

Figure 5. *Vascular labeling in the cremaster muscle: punctate deposits of carbon three days after exposure to a liver implant. (Whole-mount preparation, 80×.)*

the vascular basement membrane. Gaps in the endothelium, and some endothelial mitoses, were found in both capillaries and venules; in the leaky capillaries there was no evidence of endothelial contraction. Details will be presented in a subsequent paper.

Discussion

The purpose of these experiments was to study the effect of dying tissues on the surrounding vessels; one may ask, therefore, why we chose a model that amounts to 'pseudo infarct' rather than using the typical, focal ischemic necrosis of a visceral organ. Surely an infarct of the kidney would have shown a mass of necrosis surrounded by a zone of vascular damage; it would have been impossible, however, to determine whether this vascular damage was due to partial ischemia (as a part of the infarct itself) or to some effect of the infarct on peripheral, nonischemic tissue. Furthermore, our pseudo-infarcts are easy to replicate and allow to test tissues of any kind; they also can be used to assess the damage wrought to a parenchyma (striated muscle) by aseptic inflammation.

The initial phase of vascular leakage from the venules was expected, because venular leakage is a typical effect of vasoactive inflammatory mediators,[3,9] and a variety of such mediators should be produced in this model of 'pseudo infarct': histamine and serotonin could arise from dying mast cells of the implant; fragments of C3 and C5 could be produced by activation of complement by the necrotic tissue[17] or by nonspecific proteolytic digestion of complement proteins[18]; other potential sources include proteases from the dying tissue, plasmin, the clotting system, and platelets. This phase of venular leakage was unexpectedly mild, perhaps because of the many mediator-inactivating systems present in the blood within the well-vascularized and hyperemic cremaster. An early phase of venular leakage has been observed also after local injury.[4,19,20]

The arteriolar changes (focal arteriolar insudation) also were expected, because similar lesions had been described in rat muscle at a distance from the site of local, chronic delivery of inflammatory mediators and at the site of sterile inflammatory stimuli.[16]

The phase of capillary leakage came as a total surprise, because none of the known inflammatory mediators has such an effect. It is recognizable at 8 hours and ceases rather abruptly by the third day (Figure 5). Electron micrographs confirmed that the presence of carbon in the outer capillary wall indeed corresponds to typical 'labeling,' ie, to the accumulation of pigment particles that escaped through endothelial gaps and were retained by the basement membrane.[2] Because the carbon particles are spread fairly uniformly along each leaky capillary (Figures 2B, 3C), it is probable that many interendothelial junctions were disconnected at the same time. This is quite unlike the venular labeling induced by histamine-type mediators, which tends to be spotty. Histologically the tissue surrounding leaky capillaries was invariably inflamed, as indicated by edema and an infiltrate of neutrophils and macrophages (Figure 6C).

Previous Observations of Capillary Leakage

Vascular labeling including capillaries occurs, obviously, as a result of local trauma, and under such conditions it is immediate.[5,7] More relevant here is the delayed appearance of selective capillary labeling observed after a variety of physical, chemical, or immune insults,[4,5,7,15,21-23] the classic example being ultraviolet irradiation. In this group of lesions characterized by delayed-prolonged vascular leakage, the loss of fluid can begin to occur as early as 2 to 4 hours after mild thermal injury[6]

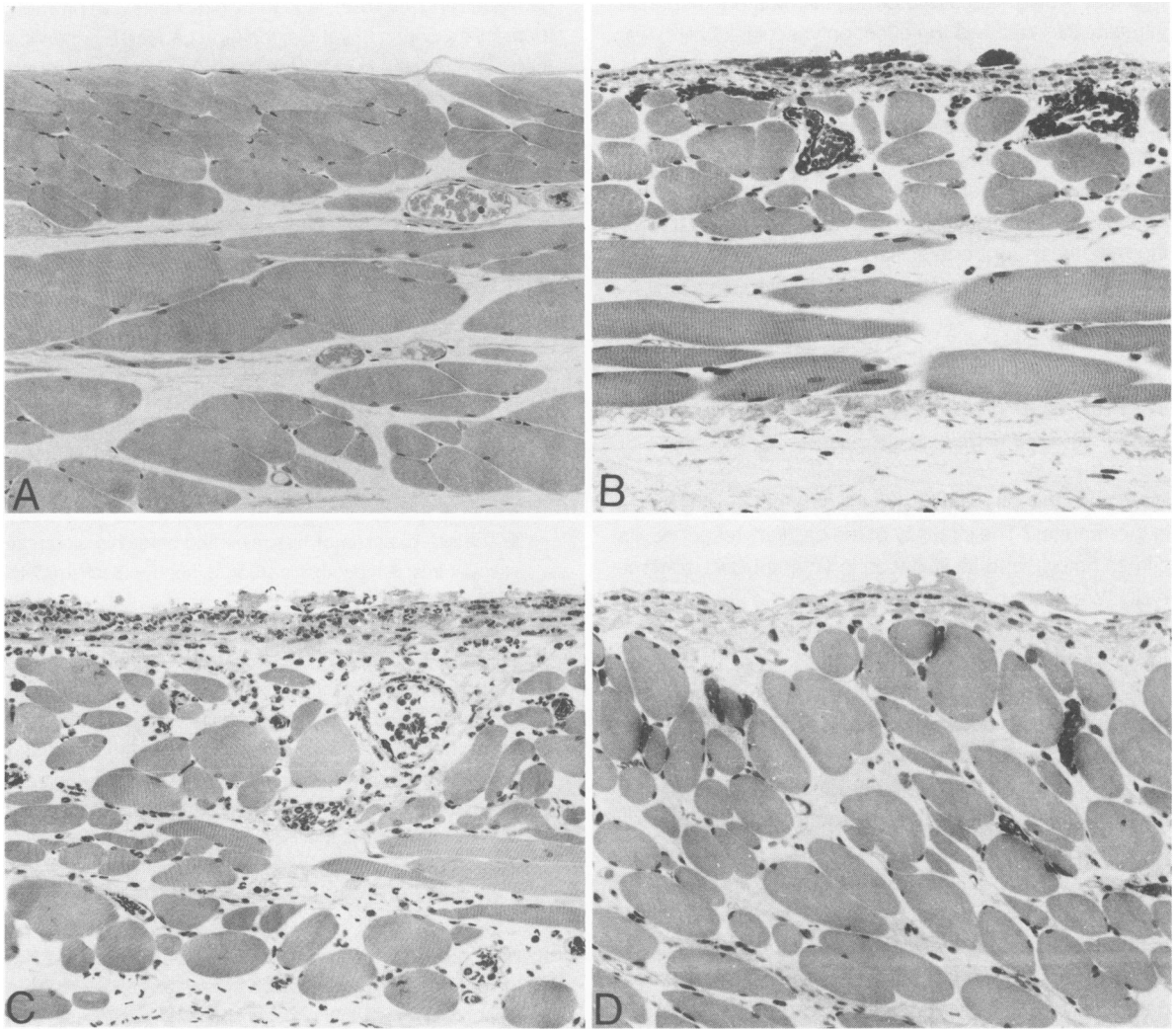

Figure 6. *Histology of the cremaster muscle (Methacrylate, H&E stain, 175×).* **A:** *Control;* **B:** *Cremaster muscle 8 hours after liver implantation: the specimen was excised from an area of heavy venular labeling. Two venules are extensively marked by carbon black; some margination also is visible. Inflammatory cells and some fibrin are present on the peritoneal surface of the muscle; deeper, edema and scattered inflammatory cells can be seen;* **C:** *12 hours after the implantation of a piece of liver the cremaster muscle is inflamed. There is some carbon deposition in the largest venule;* **D:** *18 hours after kidney implantation: labeling of most capillaries between muscle fibers. Mild edema with some inflammatory cells in the interstitium and on the peritoneal surface.*

or as late as 18 to 24 hours after local injection of a toxin[22] (reviewed by Cotran,[20] Hurley et al,[24] and Wilhelm[25]). Some of the published illustrations contrasting early/venular *versus* late/capillary damage are strikingly similar to ours.[6] The pathogenesis of these capillary lesions is not yet clear and almost certainly varies in different models. Specific mediators have been blamed, but the single explanation that stands out as very likely (at least for some forms of the delayed-prolonged leakage) is that the endothelium is directly injured, but in such a way that the damage requires several hours before it manifests itself by creating interendothelial gaps. We therefore must consider whether this explanation could apply to our results.

Necrotic Tissue as a Source of Vasoactive Materials

There is no doubt that dying tissue releases vasoactive and possibly toxic materials, as mentioned earlier, and that these materials can induce hyperemia and venular leakage; however—quite apart from the fact that no vasoactive material is known to act specifically on capillaries—the late phase of capillary leakage occurs also in the absence of dying tissue. It was present, although not as strongly, around the so-called 'controls': implants of boiled tissue, as well as of spheres of teflon or glass. We had chosen these three types of controls on the assumption

that they would cause little or no inflammatory response; in reality they did, and the effect on vascular labeling was qualitatively the same as that of the experimental tissues. This does not mean that the necrotic tissue does not contribute directly to the capillary labeling; it simply indicates that any agent capable of inducing persistent inflammation is probably also capable of inducing a late phase of capillary leakage. This must be taken as a working hypothesis, but one solitary bit of information seems to confirm it: in the rat, chronic gingivitis produces capillary leakage.[26]

Inflammation as the Cause of Delayed Capillary Leakage

How could the inflammatory reaction affect the capillaries in this manner? The intensity of the capillary response, its diffuse nature, and its limitation in time suggest some finality. We see a possible key in the mitotic activity of the endothelium, which could be interpreted as a sign of 'diffuse angiogenesis.'

A phenomenon of this nature could arise by two mechanisms, which are not mutually exclusive. 1) The cellular component of the exudate could be responsible, by producing cytokines or other mediators. The venular and capillary phases do coincide roughly with the periods of neutrophil and monocyte predominance, respectively, although not all the labeled vessels are surrounded by inflammatory cells (Figure 6D). According to this concept, the phase of capillary leakage could be the prelude to the development of granulation tissue; the leakiness of regenerating capillaries in granulation tissue is long known[27] and a correlation between mitosis and focal leakage has been observed also in large vessels.[28] 2) The hemodynamic changes related to acute inflammation could somehow induce a burst of mitoses in the endothelium, and thereby trigger a phase of capillary leakage. It is conceivable, for example, that a sustained increase in capillary pressure could cause at first a passive dilatation; thereafter the increase in endothelial cell surface could be stabilized by an increase in endothelial cell number. Both these hypotheses are testable and are currently under study.

The topic of capillary (*versus* venular) leakage has some practical implications: capillary leakage is typical, for example, of glomerulonephritis and of the adult respiratory distress syndrome,[29] in which the endothelial damage is attributed to neutrophils or to activated complement. Any therapeutic intervention against vascular leakage would have to take into account its mechanism.

To close, we will submit that the pathogenesis of microvascular leakage in inflammation is more complex than previously thought. Up to this time, three stages were envisioned: 1) immediate leakage of all vessels (arterioles, capillaries and venules) caused by direct injury; 2) promptly thereafter a phase of venular leakage caused by chemical mediators; and 3) during repair, leakage from the regenerating capillaries of granulation tissue. If our results are confirmed, there should be a period of selective capillary leakage between phases 2) and 3); current data suggest that it may be related to a form of diffuse angiogenesis.

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