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Mechanisms of Inflammation. I. Laser-Induced Thrombosis, a Morphologic Analysis.* (30393)

LESTER GRANT,† AND FREDERICK F. BECKER† (Introduced by Lewis Thomas)
Departments of Medicine and Pathology, New York University School of Medicine, New York City

It is well recognized that thrombosis in small blood vessels plays a significant role in the development of inflammatory states (1-4). The adherence of the thrombus to the vessel wall involves platelet aggregation, among other things, and the sticking of formed elements such as white cells and platelets(4) to each other and to the endothelial cell.

Recently developed laser systems(5-10) now permit a unique approach to the study of thrombosis and thromboembolism, enabling the investigator to analyze, *in vivo*, in a highly reproducible way, the formation, propagation and dissolution of microthrombi (10,11). In contrast to virtually all previously used experimental models, it is possible with a laser instrument to produce microthrombi in single vessels. One can arrange the experimental system in such a way as to exclude, apparently, extravascular factors which may influence the development of such thrombi. Under these circumstances, discrete intravascular injuries yield white cell and platelet adherence to the endothelial walls of small blood vessels. Such capillary preparations as mesenteries(10) permit short-term observations of such lesions but the rabbit ear chamber, equipped with a transparent coverslip, is an ideal preparation for long-term study of laser injury and the resolution of the lesion(11). Lesions can be observed for minutes, hours, days or weeks with the coverslip of the chamber protecting the capillary bed from extraneous stimuli, yet allowing a constant evaluation of the status of the injury site.

This communication deals with the morphology of a lesion produced by a laser beam in an ear chamber under circumstances where the injury to the blood vessel was of a sufficiently limited nature to permit recovery. Future communications will deal with the dynamic aspects of this system.

Materials and methods. Rabbits used were 2 kg male English half-lops, obtained from Bar F Rabbitry, Fullerton, Md. Ear chambers were similar in design to those described by Sanders *et al*(12) and Grant *et al*(13) except for certain modifications in coverslip support. The principle of the chamber was essentially that of Sandison(14). Connective tissue in the ear chamber is made up of subcutaneous areolar tissue and is 30 to 50 μ in thickness. The laser system used was a Ruby Minilaser 103, purchased from TRG, Inc., Melville, N. Y., adapted for use with a Leitz Ortholux microscope. The laser beam was projected by the use of a prism through the $\times$50 objective of the microscope to the target vessel. After preliminary testing of the system by production of more than 100 lesions, it was determined that a satisfactory injury in a venule could be achieved by setting the Variac on the power supply at intensities from 150 to 200 joules input. Under conditions of the experiment, the diameter of the lesion produced immediately after the stimulus *in vivo* was approximately 5 to 20 μ, which correlated well with diameters of color alterations produced by this laser beam on glass slides coated with a green dye.

Ten minutes before application of the laser beam to the ear chamber vessel, the rabbit received 5 cc of Pelikan ink in gelatin, pre-

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† Career Scientist, New York City Public Health Research Council.

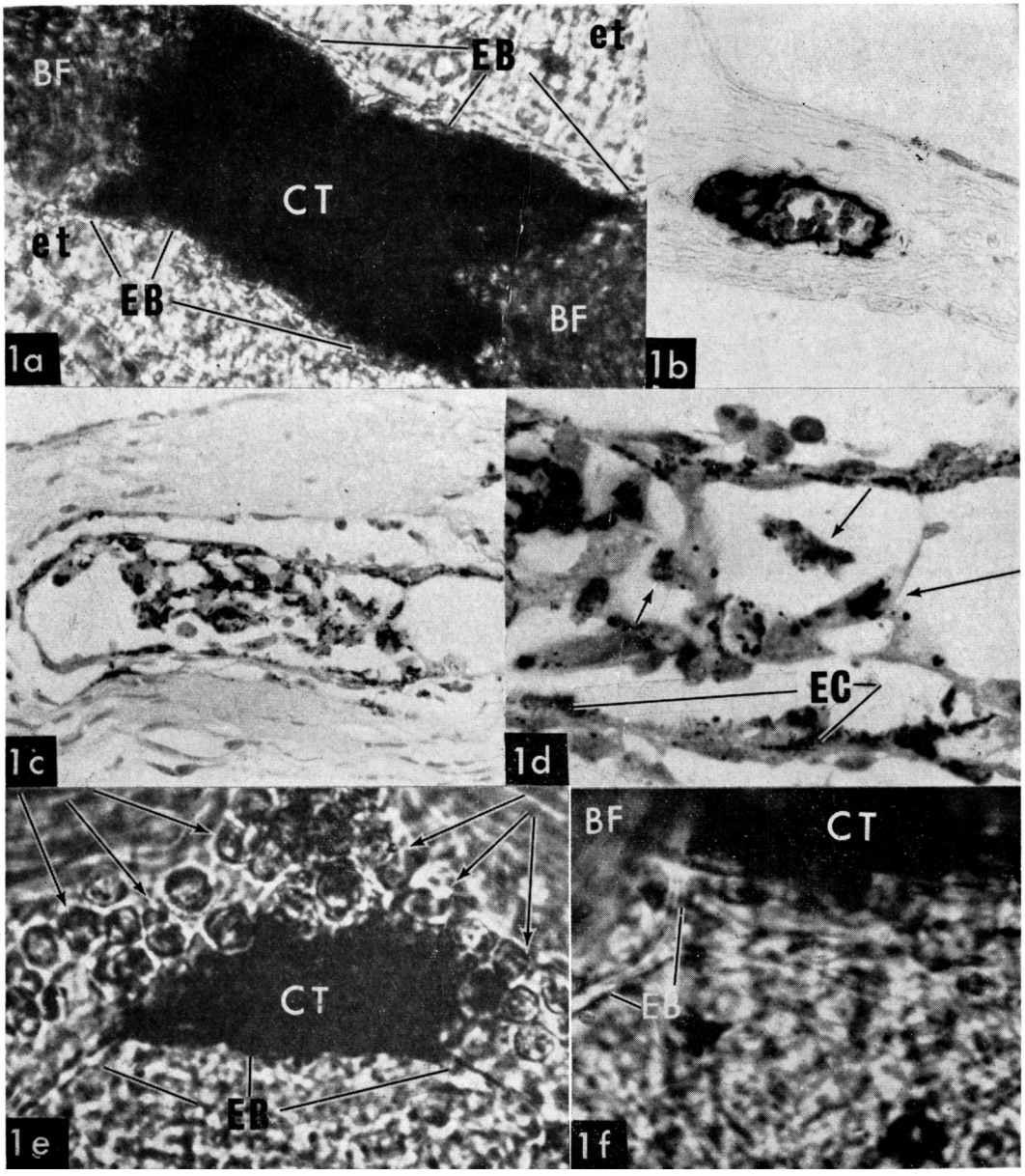

FIG 1 a. Photograph of a venule with a laser lesion *in vivo*, showing carbon sticking at 3 hr. Note blackening of lesion, rather well defined endothelial borders, clear extravascular tissue and blood flow proximal and distal to the lesion. CT = carbon thrombus; BF = blood flow; EB = endothelial border; et = extravuscular tissue. × 210.

FIG. 1 b. Microphotograph of the thrombus shown in 1 a. This picture is a cross-section of thrombus, cut in 3 μ sections after fixation of the tissue. Bridging of vascular lumen has resulted in partial occlusion of the vessel. Carbon apparently is deposited on entire endothelial surface but the vessel (as indicated in 1 a, an *in vivo* photograph) is not totally occluded. H & E × 21.

FIG. 1 c. A similar 3-hr lesion. The overall form is a cribriform mass bulging in lumen. Entrapped in thrombus are white blood cells. There is some artifactual separation of endothelium and basement membrane. Carbon is seen deposited on the white blood cell and amorphous material of thrombus, endothelial cells, basement membrane and pericytes. H & E × 105.

FIG. 1 d. High-power microphotograph of lesion illustrated in 1 c. Arrows point to white

pared according to the formula of Biozzi(15).

Observations were made *in vivo* virtually continuously for periods up to one hour and at 15-minute intervals thereafter in the three-hour experiment. All animals were placed in the supine position during the experiment, and were conditioned to this position, making anesthesia unnecessary.

At the conclusion of *in vivo* observations, the chamber tissue was fixed and stained according to the following procedure. After removal of the protective coverslip of the ear chamber, fixative was applied throughout the remaining maneuvers. The chamber tissue and surrounding cartilage were cut from the rabbit's ear and placed in a Petri dish containing fixative. Under a dissecting microscope and using a razor blade, the tissue was freed from cartilage and then one edge (that closest to the lesion) was squared by a single cut approximately 0.5 mm distal to the lesion. The tissue was carefully pinned to a small square of rough cardboard using dehubbed 30-gauge needles and fixed for one hour.

The fixative used throughout the experiment was reagent grade 10% formaldehyde, phosphate buffered to pH 7.2, containing 7.5% sucrose. Following fixation, the tissue was dehydrated by hand passage through alcohols at 20-minute intervals. Incubation in paraffin for 18 hours was followed by blocking the tissue in standard paraffin boats utilizing fine-tipped jeweler's forceps to insure placing the flat edge against the base of the boat. Sections were cut at 3 μ on a standard microtome, the blade of which had been sharpened immediately prior to use.

The stains utilized included H & E, PAS, PTAH, Alcian Blue and a standard basement membrane silver stain(16).

Tissue for histologic sections was obtained at 3 hours and at 24 hours.

Observations. 1) In vivo *observations.* Under the conditions of the experiment, no lesion was discernible with the laser beam if the animal did not receive carbon prior to the stimulus, although it was possible, with higher intensities of stimulus, to achieve a thrombus without use of carbon. With the injection of carbon, there was a darkening of the animal's blood in the ear chamber but no sticking of carbon to endothelial surfaces, nor was there any stigmata of inflammation caused by the injection of carbon. The phenomenon noted by Stehbens and Florey(17)—the clumping of carbon in masses of white cells and platelets —was not apparent in these preparations except at the specific site of injury. The microscope ocular was closed during the pulsing of the laser stimulus so that laser energy would be confined to the target. Immediately (within a second or two) after the laser stimulus, the ocular system was opened and a lesion in the target venule was apparent. This lesion consisted of a small (5 μ) clump of red cells, surrounded by white cells and platelets which were stuck at the injury site. At this time, no leakage of carbon was noted, no white cells had emigrated into the extravascular spaces and there were no perivascular morphological alterations. Over the next 3 hours, the thrombus enlarged by addition of white cells and platelets but not by addition of red cells. Intermittently the thrombus grew to a maximum size of 40 to 50 μ, achieved at approximately 3 hours (Fig. 1 a).

cells caught up in amorphous material in lumen of vessel. EC = endothelial cells. It would appear that there has been phagocytosis of the carbon by both white blood cells and endothelial cells. H & E × 315.

FIG. 1 e. Photograph of a venule with a laser lesion, *in vivo*, taken at 5 hr with a less heavy deposit of carbon than shown in Fig. 1 a. Arrows point to white blood cells which have affixed themselves to the injury site. The shutter on the camera was set at 1/5 sec. This caused the red cell flow (at top left and top right of photograph) to appear as a blur but the white cells appear to be in focus because they were relatively stationary. EB = endothelial border; CT = carbon thrombus. × 210.

FIG. 1 f. Photograph of the same venule taken at 5 days showing a disappearance of the carbon from injury site. Endothelial border on the left is the same as endothelial border in 1 e although the photograph covers more of the extravascular tissue in order to show 2 extravascular clumps of carbon, both of them apparently cell-bound. One of these is to right of marker "EB," the other in lower right-hand corner. The carbon thrombus is deliberately out of focus in order to focus on the extravascular clumps. BF = blood flow; CT = carbon thrombus; EB = endothelial border. × 280.

The intermittency of growth was the result of occasional breaking off of clumps of white blood cells and platelets which were carried off in the direction of blood flow. During the 3-hour period there was a progressive darkening by carbon of the injury site, reaching a point at 3 hours where the thrombus was homogeneously jet black. At this time the cellular components of the thrombus were obscured. Although the growth of the thrombus interfered with blood flow, there was no total shutdown of flow at any time. Over the next 21 hours, a typical course of resolution was noted in all animals studied. There was a progressive diminution of carbon adherence and in the size of the thrombus, and a progressive increase in the volume of blood flow past the site of the lesion. At 24 hours, the typical lesion consisted of a small aggregate of carbon and amorphous material. Carbon marking of endothelium could not be seen under highest powers of the light microscope, including oil immersion magnifications of approximately 1000 ×.

During this 24-hour period, the endothelial wall seemed to remain intact, there was no escape of red cells at any time, and there was no obvious emigration of white cells, but rarely there were small clumps of carbon seen extravascularly, apparently within macrophages. In a preparation followed for 5 days, carbon extravasation was also rare; indeed, in the perivascular area there were only 2 small clumps of carbon noted, both of these seeming to be cell-bound, perhaps in macrophages (Fig. 1 e, 1 f). The bulk of the carbon apparently had been washed away by blood flow and the remaining deposit at the injury site seemed to be rather discretely marginated, presumably held by the basement membrane.

2) *Histological observations.* The term "upper endothelial surface" used below refers to that surface of the vessel closest to the microscope objective during laser exposure. The appearance of the chamber tissue in histologic section is that of a delicate, collagenous membrane containing vascular channels. The connective tissue matrix is composed of unidirectional collagen bundles with minimal cellularity. Occasional fibroblasts are present and extremely rare mononuclear cells are also seen. The vascular spaces are simple in form, composed mainly of tubes lined with endothelium with an extremely thin basement membrane, visualized in silver stains. Around the larger vessels, there is a suggestion of condensation of collagen. Pericytes are rare and only detected about the largest vessels. Occasional lymph channels adjacent to the major vessels are identifiable by their thin-walled structure, moderate lumen size, lack of basement membrane and lack of erythrocytes.

Neither at 3 hours nor at 24 hours was there a perivascular inflammatory response in the area of the damaged vessel. At most, rare mononuclear cells were noted outside of the pericytes at 24 hours, and these occasionally contained carbon particles. No free carbon was seen beyond the basement membrane at any time and the source of these macrophages and their carbon content was not obvious. At 24 hours there could be seen a granular quality to the collagen above and below the affected vessel.

The thrombi were entirely within the endothelial tube. At their most central portion, they occasionally bridged the entire lumen but did not totally occlude it. Serial sections of the entire thrombus were utilized routinely and demonstrated an overall spindle form. The propagated edges were usually adherent to the upper endothelial surface. The total length of the lesions as determined by serial sections ranged from 40 to 60 μ.

At 3 hours, the thrombus consisted of a cribriform mass of white blood cells, platelets, amorphous material, occasional red blood cells and masses of carbon (Fig. 1 b, 1 c). The carbon was entrapped within the thrombus and was also distributed across the surfaces of the thrombus and its component cells. The endothelial cells could be identified and appeared swollen. Carbon was apparent as both a surface deposit and also seemed to be phagocytized by the endothelial cells (Fig. 1 d). No intervening material could be identified between the carbon and those endothelial cells upon which it was deposited. The propagated margins of the thrombus were composed of masses of carbon in an amorphous matrix adherent to an apparently normal endothelium.

There was an artificial separation of the thrombotic mass from the underlying basement membrane in some sections. The basement membrane, however, appeared to be intact. At 24 hours, carbon was deposited along the basement membrane underlying the thrombus and there was also a suggestion of some carbon present in the pericytes at the same location.

By 24 hours, the lesions assumed a more compact form, with elimination of the cellular elements. The residual thrombus was composed of aggregates of carbon particles and amorphous material adherent to the upper endothelial surface. Occasional channels containing red blood cells, which were rare at 3 hours, coursed through the thrombus at 24 hours. At 3 hours the amorphous material was faintly PAS positive and there was a suggestion of a faint purple coloration of the same material with PTAH.

Discussion. The laser (light amplification by stimulated emission of radiation) is an artificial light source capable of a narrow focal diameter which produces a localized thermal effect upon absorption by an appropriate target(5,8). This dependence upon absorption for the generation of heat and local effect was demonstrated by the failure to produce a lesion of any sort under the conditions of this experiment when the laser beam was applied to the ear chamber of animals which had not received carbon. It is apparent, therefore, that the carbon in the blood stream acted as a site for heat generation, presumably causing a microburn on the luminal side of the vessel wall. It is possible, however, to achieve such lesions without the use of carbon or dyes; one then loses a marker for the lesion and runs the risk of damaging extravascular tissue.

In addition, it is possible that the local generation of heat, occurring in a microsecond, fused components of the blood to the endothelium, resulting in the microthrombus which is noted to be present instantaneously after the laser beam application. That the effect is entirely intraluminal is indicated by the findings that (1) there is no extravasation of blood, white cells or carbon and (2) that there appears to be no visual discontinuity of the vessel wall. It is intraluminal in the sense that the lesion is confined to the inside of the vessel, that is on the luminal surface of the endothelium and does not appear to extend extravascularly. Because the thrombus does not move, it must have a vessel wall attachment; otherwise the pressure of blood flow would cause it to move downstream. In addition, there is no evidence of a perivascular inflammatory response, but it should be noted that the histological sections raise the possibility of collagenous degeneration near the site of the lesion. Whether this is an artifact of the tissue processing under these conditions, and therefore a spurious finding, or a true lesion will be discussed in a report of the electron microscopic aspects of this study.

The enlargement of the thrombus results from the adherence of white blood cells and platelets to the original microthrombus. The nature of this adhesiveness—either the original sticking to the endothelium or the growth of the thrombus—is not understood. All efforts to define the character of an imagined "glue" which causes intravascular sticking have failed to yield an agreed upon mechanism(11). It would appear that stickiness can be imparted either by injured endothelium, or injured white cells or platelets or perhaps by some component of the blood which is altered in inflammatory states. There is no evidence from these experiments to suggest that "glue" exists between the thrombus or its carbon coating, and the endothelial cell, but is unlikely that the magnifications used in these preparations would reveal such material, assuming that it is in fact present.

The question of why white cells stick to the endothelial wall in a wide variety of inflammatory states and red cells do not stick under the same circumstances, as in the reactions reported here, has long intrigued investigators(11). Bangham *et al* opened an experimental approach to this problem with the suggestion that the differential adhesive properties of blood cells might be attributed to the presence on their surfaces of different ionic species, yielding an hypothesis that could account for differences in cellular adherence to endothelial walls(18).

The fact that carbon is necessary for the evolution of the lesion indicates that the de-

velopment of the thrombus is directly and specifically related to the presence of carbon in the blood stream. This could place the origin of such thrombi in a narrow category of experimental irrelevancies, unrelated to biological phenomena. But, as has been pointed out(11), laser lesions are produced not only with carbon, but with Alcian Blue, a mucopolysaccharide dye, and Evans Blue, a dye unrelated either to carbon or Alcian Blue in chemical structure or physiological characteristics(19). A preferred hypothesis is that any material which can absorb the energy of laser will transmit a heat injury intravascularly. The significant point appears to be that the arrangement of the system in such a way as to achieve a lesion with a dye or a colloidal material, and the failure to do so without such material, provides something of a guarantee that the injury will, in fact, occur on the luminal side of the endothelial wall. This indicates that the extravascular tissue, including collagen and its cellular components, and the basement membrane are transparent to the laser beam. The laser injury in ear chamber tissue thus becomes a unique tool for studying the pathogenesis of intravascular pathology, for the system eliminates extravascular factors which often intrude on intravascular inflammatory reactions because of the relative diffuseness of many experimental stimuli. It is perhaps significant that there was no emigration of white cells, or a very limited emigration, despite the flagrant intravascular pathology and there was very little carbon "leaking." It may not be unreasonable to assume that the pathogenesis of "leaks"(20-22) are related as much, or more to extravascular factors, such as chemical mediators, as to endothelial injury *per se*. These experiments raise this issue; they do not settle it.

Even more complex, perhaps, is the nature of the resolution of the lesion. There comes a time when carbon sticking abates, within hours as these experiments were performed. The diminution of carbon sticking might be explainable in part on the grounds that the lesion is no longer exposed to carbon due to its clearance from the blood stream. In one preparation followed for 8 days, a gradual disappearance of the carbon at the site of the lesion was noted (Fig. 1 e, 1 f) with an uncovering of what appeared to be normal endothelium, that is, a return to the status of the chamber vessel before injury. It would appear that the carbon disappeared under the "erosion" of the forces of blood flow. But whereas carbon may diminish in the blood stream to be sequestered in the liver and spleen, neither white blood cells nor platelets diminish in a comparable way—indeed, presumably they do not diminish at all—and the lesion's exposure to these elements is unchanged as long as there is vessel flow. This would suggest that there is another factor at work in the blood stream, a factor relating to the alterations of cell surfaces in such a way as to convert injured surfaces from sticky to non-sticky states. Such a factor has long been sought(11). Again, these experiments raise the point but, at a morphological level, do not define it.

A provocative finding was the observation, in fixed sections, of apparent phagocytosis by endothelium of carbon particles at 24 hours. This adds more evidence to a long-standing argument as to whether endothelium has phagocytic properties for macromolecules(23, 24). Although under the magnifications of the material presented here, the issue cannot be settled, it would appear that carbon particles are phagocytized by endothelium in this type of heat injury. A recent discussion of the problem by Cotran(25) indicates that certain specific circumstances (*e.g.*, "overloading" of the animal with colloidal carbon) led to phagocytosis by non-reticulo-endothelial vascular endothelium.

Summary. 1. A small injury has been created with a laser beam in a venule of a rabbit ear chamber. The injury is caused by the absorption of light by carbon particles injected into the blood stream of the rabbit and the subsequent generation of heat. 2. This injury results in formation of a thrombus in the vessel without apparent intervention of perivascular, or extravascular, factors. 3. The thrombus grows by the adherence of white cells and platelets to the lesion over a period of hours. 4. Resolution of the lesion results from a failure of further sticking of these elements, suggesting the existence in the blood stream of some "anti-sticking" mecha-

nism, possibly in conjunction with the normal "erosive" properties of red blood cell flow. 5. The morphologic characteristics of the injury *in vivo,* and by histologic sectioning have been described. 6. There is evidence that the endothelial cytoplasm may have phagocytic properties under the conditions of the experiments described here.

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Serum Beta-Glucuronidase Activity of Various Species: Possible Correlation with Susceptibility to Atherosclerosis.* (30394)

BENJAMIN F. MILLER, FRANCES P. KEYES, P. WILLIAM CURRERI
Harrison Department of Surgical Research, University of Pennsylvania, Philadelphia

Beta-glucuronidase activity has been found to be increased in human atherosclerotic arteries by Dyrbye and Kirk(1) who studied intima-media portions of the aorta and coronary arteries, and also by Branwood and Carr(2) who assayed the intimas of coronary arteries. In cholesterol-induced atherosclerosis in the rabbit, Mrhova *et al*(3) observed that the enzyme activity was also increased in whole aortic extracts. On the other hand, Kayahan(4) reported decreased values for beta-glucuronidase activity in the intima of human, atherosclerotic aortas.

Kayahan noted briefly(4) a high value for

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