On the Mechanism of Vascular Leakage Caused by Histamine-Type Mediators

A MICROSCOPIC STUDY IN VIVO

By Guido Majno, M.D., Virginia Gilmore, and Monika Leventhal

ABSTRACT

A current hypothesis maintains that the vascular leakage induced by histamine, serotonin, and bradykinin is brought about by a hydrostatic mechanism: The larger veins are said to contract; the venules are therefore submitted to an increase in pressure, which causes their wall to give way. To test this hypothesis, vessels of an exposed striated muscle (rat and rabbit cremaster) were photographed while treated with the above-mentioned substances; the occurrence of vascular leakage was tested by the intravenous injection of carbon black (carbon labeling). All animals were under barbiturate anesthesia, given a neuromuscular blocking agent (gallamine triethiodid), and were maintained by artificial respiration, according to a technique previously described. In 72 rats and 4 rabbits, the first event observed was arteriolar dilatation. Constriction of a small vein was observed in only 1 in 76 experiments (in two other cases it was doubtful). It is concluded that venous spasm cannot account for the leakage induced by the mediators of the histamine type, though such leakage may be enhanced by venous spasm whenever it may occur. An alternative mechanism is proposed and discussed: Histamine-type mediators cause vascular leakage by a direct effect on the venular endothelium, which is induced to contract.

ADDITIONAL KEY WORDS

permeability exudation serotonin bradykinin microcirculation inflammation venoconstriction endothelial contraction cremaster muscle rat rabbit

Histamine is the prototype of a group of endogenous chemical "mediators" which induce blood vessels to leak. Though chemically dissimilar, these compounds may be justifiably considered as a group (1) because they have certain pharmacologic effects in common: they cause blood vessels to leak; this effect occurs within seconds of their application, is brief (lasts for about 15 min)¹ and occurs primarily in venules (2).

It is established that the leakage occurs through submicroscopic gaps that appear between the endothelial cells (4). The next step, therefore, should be to find out how the chemical mediator brings about the intercellular gaps. Until quite recently only one plausible hypothesis (5) was available: It explained the formation of the gaps by a purely hydrostatic mechanism, i.e., by an increase in pressure caused by a constriction of the veins. The latter phenomenon has long been assigned (6-8) a role in the physiology of histamine and also long debated (9, 10); it is obvious that if the larger veins contract, the blood pressure in the venules is raised; and it has been proposed by Rowley (5) that the venules may actually "burst" under the raised hydrostatic pressure, that is, their endothelial cells become dissociated.

This hypothesis is attractive in several respects. It offers a reasonable explanation for the preferential effect on the venules (ac-
according to the law of Laplace [5] the tangential force tending to disrupt the wall decreases with the radius of the vessel) and makes it easier to understand why many of the mediators that cause blood vessels to leak should also be substances that cause smooth muscle to contract.

We therefore attempted to confirm this hypothesis in a series of experiments in which we raised the venous pressure in the hind limb of the rat with the use of a cuff (11). However, the vascular leakage produced in this manner was minimal, and bore no resemblance, quantitatively, to that of the histamine-type mediators. Thus we decided to reinvestigate the alleged venous spasm by studying the blood vessels in vivo under the effect of the mediators in question. The first requirement for a study of this kind was a preparation of live tissue which would be rich in blood vessels, including a wide range of calibers, lying as closely as possible in the same plane, and allowing good photographic resolution. Since the rat cremaster seemed to fulfill these characteristics, we first devised a method for the study of this muscle in vivo (see preceding paper) (12). The present report deals with the effect of histamine, bradykinin and serotonin as studied on the blood vessels of this preparation.

Material and Methods

We used 72 rats of the Sprague-Dawley strain mostly in the range of 180 to 230 g (141 to 315 g), and 4 albino rabbits weighing 1890 to 2470 g. Twenty other rats were used but discarded for various reasons (12). Each rat was anesthetized (sodium pentobarbital 6 mg/100 g ip) and its cremaster was exposed as described (12). All animals were treated with a neuromuscular blocking agent (12). The test substances, dissolved in phosphate-buffered Krebs-Ringer medium, were: histamine phosphate (E. Lilly and Co.), 1 mg/ml; bradykinin triacetate (Sigma Chemical Co.) prepared in various strengths immediately before use, or frozen and then thawed at the time of use; and serotonin creatinine sulfate (Nutritional Biochemicals Corp.) 0.1 mg/ml. All concentrations are calculated for the salts. The concentrations actually reaching the blood vessels were presumably somewhat lower than those just mentioned; though most of the Ringer solution was pipetted off and then replaced with an equal amount of test solution, some dilution inevitably occurred in the residual fluid overlaying the cremaster and in the hydrated connective tissue surrounding it. The cremaster was under visual microscopic observation throughout, and records were taken either on 16-mm color motion pictures, or on 35-mm still photographs in color or black and white.

Experimental Procedure

The experimental protocol was as follows. The animal lay anesthetized on the special table, with a cannula in the jugular vein (connected to a syringe containing carbon black) and the cremaster exposed under a shallow pool of solution. An appropriate field was selected through the microscope, and carbon black was then injected iv. The subsequent timing was critical, and took into account the following facts: (A) the purpose was not only to study the caliber of the veins, but also to identify leaking vessels by labeling with carbon; (B) pilot experiments had shown that the entire episode of labeling, as induced by histamine-type mediators, can be considered accomplished in 2 to 5 min (see also ref. 5); (C) carbon black is cleared very fast from the circulation; it can effectively label leaking vessels for about 5 min after a single injection, but thereafter the injection must be repeated; (D) if any vessel has been damaged by the surgical procedure, it will be labeled within the first 30 sec after the carbon injection; (E) the exposed cremaster can be maintained without significant damage for at least 1 hr (12, 13).

From these considerations the following protocol emerged: (1) intravenous injection of carbon; (2) waiting period of 30 sec to 2 min, to allow time for possible surgical damage to reveal itself through vascular labeling (if appreciable labeling developed, another field was selected or the preparation was discarded); (3) dripping of the test substance onto the cremaster: Labeling then occurred, and observation continued until it was completed, usually no longer than 6 to 7 min from the carbon injection.

Occasionally a variant of this protocol was adopted to provide an even stricter control of the surgical damage: (1) carbon injection, (2) waiting period of 3 to 60 min, (1a) new carbon injection, (2a) further waiting period of 1 to 2 min, (3) application of the test substance.

To study how the previous degranularion of the mast cells might affect the results, 8 rats were injected ip 5 days prior to the experiment with 48/80 (Burroughs Wellcome and Co.), 1 µg/gm body weight, in 10 ml of Ringer's fluid; no significant difference was found. Of the rabbits, 1
was tested with histamine and 3 with bradykinin (0.1 and 0.01 mg/ml). Table 1 summarizes all the experiments on rats.

**Terminology.** In a given organ, vessels of the arterial and venous trees respond differently to the same stimulus, according to their caliber and to their position in the vascular tree. The two factors are obviously interrelated. We prefer to link our terminology to the vascular tree, because the orders of branches are more immediately apparent in our system, and because the fine arterial branches are difficult to calibrate with any accuracy in view of the wide range of functional changes. Thus, we shall use the following conventions: small arteries—all arterial vessels excluding the last 3 to 4 orders of branches; arterioles—the last 3 to 4 orders of branches, including the precapillary vessels; capillaries—the smallest vessels; venules—the first 3 to 4 orders of postcapillary vessels; and small veins—the next vessels downstream (rarely exceeding 100 to 150 μ in the rat cremaster).

**Results**

The results were almost identical with all three mediators, with mainly quantitative differences. Bradykinin gave the most intense vascular labeling and serotonin gave the weakest at the concentrations tested.

**Observations on Rat Cremasters**

Small arteries. In most cases, no change in caliber was observed; in 9 instances (and never with histamine), a chain of irregular circular constrictions appeared immediately after application of the test substance; this spasm wore off in 10 to 15 sec and was followed by dilatation.

Arterioles invariably dilated under the effect of the chemical mediators. This was the first and unerring effect of the three substances tested, in agreement with the study of Ebert and Graham on rabbit ear chambers (14). At the beginning of the experiment arterioles were barely visible; the injection of carbon black brought them temporarily into prominence by their black contents; within seconds after the application of the mediator they increased their diameter two- or threefold (Figs. 1 and 2).

Capillaries. A characteristic flush appeared within seconds after the application of the mediator; this corresponded, primarily, to an increased perfusion of the capillary net.

Venules. Three almost simultaneous changes were observed in these vessels 10 to 30 sec after application of the chemical agent: (A) an increase in the rate of flow, detectable from the speed of the red blood cells; (B) a moderate increase in caliber (Figs. 1-5), and (C) vascular labeling. Of these three, only the increase in caliber was inconstant.

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**Table 1**

<table>
<thead>
<tr>
<th>Test substance</th>
<th>Waiting period*</th>
<th>Number of rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histamine (1.0 mg/ml)</td>
<td>30 sec–1 min, 45 sec</td>
<td>18†</td>
</tr>
<tr>
<td></td>
<td>2 min–2 min, 30 sec</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>5 min–10 min</td>
<td>2‡</td>
</tr>
<tr>
<td></td>
<td>8 min–15 min</td>
<td>4‡§</td>
</tr>
<tr>
<td></td>
<td>20 min–25 min</td>
<td>2‡</td>
</tr>
<tr>
<td>Bradykinin (0.05 or 0.01 mg/ml)</td>
<td>1 min–1 min, 20 sec</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>2 min–2 min, 30 sec</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>3 min–6 min</td>
<td>2‡</td>
</tr>
<tr>
<td></td>
<td>8 min</td>
<td>4‡§</td>
</tr>
<tr>
<td></td>
<td>22 min–60 min</td>
<td>6‡</td>
</tr>
<tr>
<td>Serotonin (0.1 mg/ml)</td>
<td>45 sec–2 min</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>60 min</td>
<td>1‡</td>
</tr>
</tbody>
</table>

*Between intravenous carbon and local application of mediator.
†Two of these animals were used for the study of fascial vessels only.
‡A second intravenous injection of carbon was given 1 to 3 min prior to the test-substance.
§Animals prepared by ip injection of 48/80 five days previously.
The labeling appeared in the form of tiny black deposits, first granular, then confluent, on the wall of the venules. By studying the profile of these vessels at higher powers one can see that the deposits are actually forming "outside" the wall (though in effect they are still within the basement membrane) (4) (Figs. 4 and 5). A fully developed carbon

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Effect of bradykinin, 0.01 mg/ml, on rat cremaster. (1) 26 min after a first injection of carbon, a second one was given; 40 sec later, no vessels are labeled. Note caliber of arteriole A. (V = venule; large vessel is a vein.) (2) 20 sec after bradykinin (and 3 min after the second carbon injection). The arteriole has greatly dilated. (3) 50 sec after bradykinin. Arteriole is still dilated; venule V has begun to label. (4) 66 sec after bradykinin. Venule V is strongly labeled. The large vein has not become constricted throughout the sequence.
Sequence illustrating venular labeling after histamine, in the absence of venous contraction, and with minimal venular dilatation. (1) 6 sec after carbon. Note laminar flow (x) in small vein. The carbon is unevenly distributed. (2) 11 sec after carbon. Laminar flow (x) is now apparent in other vessels. (3) 2 min after carbon. The blood is dark, but no venular labeling has developed. (4) 4 min, 5 sec after carbon and 1 min, 35 sec after carbon.
deposit therefore appears as a cuff around the vessel. At first sight, this may suggest that the venule is constricted on both sides of the cuff, a misinterpretation that is easily avoided by studying sequential frames. For the same reason, an intensely labeled venule looks larger than its lumen would warrant, its wall being thickened by the carbon (Fig. 5). Occasionally, and then more often with histamine, labeling only became visible 1 min after application of the drug. Black parietal thrombi (small masses of carbon enmeshed in thrombi, and which adhered temporarily to the vascular wall) and were then washed away, were observed on rare occasions.

Small veins. In over 50 consecutive experiments with all three drugs, no change in caliber was observed in these vessels (Figs. 1-7). In a single case out of the 76 animals tested a small vein went into spasm as soon as the bradykinin was applied (0.1 mg/ml) (Fig. 8); the spasm wore off in 2 min. The caliber of this venule was -90 μ (see Discussion). In two other cases a vein of similar diameter showed a questionable focal narrowing which could not be distinguished from an artifact due to a change in depth of the photographic focus.

Observations on Vessels of the Fascia

For simplicity we are also including under the term “fascia” in the rat the long, flat, villiform connective tissue structures that wrap the cremaster (12). The changes observed were the same as in the cremaster, except that arteriovenous anastomoses were obvious and frequent; in these cases the anastomosis (a vessel considerably larger than a capillary) behaved with regard to leakage as might be expected (2): It became labeled only on the venous side.

Observations in the Rabbit

Results were the same as in the rat, except that arterial spasm was not observed, and that the carbon black (even if injected slowly) did not appear to mix evenly with the blood (12) but formed microscopic emboli which occasionally became impacted (Figs. 6 and 7). The capillary emboli appeared as thin, threadlike black segments, quite different from the granular deposits of true labeling. They correspond to the “plugs” described earlier (1). Those which were followed slowly worked their way through the capillary (Fig. 7).

Discussion

This series of experiments was undertaken to find out whether the venular leakage caused by histamine-type mediators may be explained by venous spasm downstream from the venules. The question is of considerable importance, since it concerns a key phenomenon in the process of acute inflammation. The results of our experiments are in opposition to the only other study in the field (5)—venular leakage occurred without any visible change in caliber of the small veins. Our discussion, therefore, will be subdivided as follows: (1) reliability of our results, (2) analysis of the conflicting data from the literature, and (3) proposal of a new and truly “conciliatory” hypothesis to explain the vascular leakage induced by histamine-type mediators.

Reliability of Our Results

Our main observations can be summarized by stating that the three mediators tested, in 73 (and possibly 75) of 76 experiments, caused no venous constriction. In this respect our results agree with those of Grant on the rat cremaster (13), of Taichman and Goldhaber on the hamster cheek pouch (14) and of Ebert and Graham on the rabbit ear chamber (15), who never observed venous constriction. The first objection that might be raised is that spasm could have occurred in a vein not included in the field of observa-

histamine. Venular labeling is apparent. (5) 3 min, 30 sec after histamine. The labeling is stronger. (6) 5 min, 30 sec after histamine. Labeling is as strong as it will become. Note that throughout this sequence some venules (arrows) label with no change or only minimal increase in their diameter; heavy labeling (6, lower arrow) gives a misleading impression of dilatation.

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FIGURE 4

Effect of bradykinin, 0.01 mg/ml, on rat cremaster. (1) 2 min, 8 sec after carbon. Arrow points to a leukocyte "rolling" along the wall of the small vein. V = venule. (2) 5 min after carbon; decreased perfusion of the capillaries during a "resting phase." (3) 23 min after the first injection of carbon a second one was given: 1 min, 45 sec later, there is no vascular labeling at all, circulation is lively. (4) 2 min, 15 sec after the second carbon
FIGURE 5

Effect of bradykinin, 0.01 mg/ml, on rat cremaster. (1) Normal circulation. (2) 15 sec after carbon. Note laminar flow (x). (3) 35 sec after bradykinin and 3 min after carbon. No labeling yet. Some branches of the venule have dilated, others not visibly so. (4) 1 min, 15 sec after bradykinin. The branch which has dilated the least (arrow; compare with frame 2) has labeled the most. (5) 2 min after bradykinin. There is strong labeling. (6) 4 min, 5 sec after bradykinin. Labeling is completed. Note that carbon deposits appear to form a cuff around the venules.

This is most unlikely. In the vast majority of our experiments, the microscopic field was specifically selected so as to include the largest vein visible. This being the case, one could still object that our observations—purposely done at relatively low powers to include a whole segment of the venous tree—limit our resolution, so that a reduction in vascular diameter of 5% or less would escape notice. This is true, but then it would be unreasonable to maintain that such a small change in caliber could represent the basic

injection. There is still no vascular labeling. Arrow points to some "rolling" leukocytes. (5) 4 min after the second carbon injection, and 1 min, 45 sec after bradykinin. Strong labeling develops and spreads along the venous end of capillaries (arrows), as often occurs with bradykinin. (6) 4 min after bradykinin. The labeling is completed. Arrows point to "cuffs" of carbon in the wall of a venule.

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Effect of bradykinin, 0.01 mg/ml, on the fascial vessels overlying the rabbit cremaster. F = fatty tissue. The vein V was the largest exposed. (1) 25 sec after carbon. Note caliber of the two venules indicated by arrows. A small carbon embolus, as often seen in rabbits, impacted in an arteriole (small arrow). (2) 1 min after bradykinin and 2 min, 41 sec after carbon. Note small embolus E traveling along an arteriole (it disappears in the subsequent frame). (3) 1 min, 40 sec after bradykinin. There is strong labeling. The caliber of one of the labeling venules (top arrow) is practically unchanged. (4) 5 min, 10 sec after bradykinin. Labeling is completed.

mechanism of a drastic, all-or-none event as vascular leakage. Finally, we did observe a definite venous spasm in 1 of 76 cases. This observation, truly baffling in its uniqueness, is made all the more intriguing in that it concerned a vein so small (~90 μ) that it

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Effect of bradykinin, 0.01 mg/ml, on rabbit cremaster. (1) 2 min, 39 sec after a slow (1 min) injection of carbon. Oblong emboli (E) are seen in a capillary, a typical event in the rabbit. (2) 11 sec later. The embolus (E) has advanced almost 1 mm to the curve of the capillary. (3) 1 min, 15 sec after bradykinin and 6 min, 2 sec after carbon. The embolic masses have become compressed into one and pushed a little further. The venule and its branches are becoming labeled. (4) 4 min, 45 sec after bradykinin. The embolus has broken up somewhat; other, smaller emboli can be seen (arrows; they are not present in the adjacent frames). (5) 7 min after the first frame. The embolus E has much disintegrated; venular labeling is well advanced (not well visible in the photograph against the very dark blood).

Analysis of the Conflicting Data from the Literature

The arguments which have been published (5) in support of the vено-spastic concept are principally the following: (A) Constriction of veins was observed in everted skin flaps of the living rat. We repeated these experiments and also obtained transient contractions of “large” veins (calibers of the order of 500 μ). Veins of this size, however, do not exist in the rat cremaster. Using his-

may have had no smooth muscle with which to contract. We will return to this observation under the last section discussed.
The single instance, in 76 experiments, in which venous constriction occurred (bradykinin, 0.1 mg/ml): (1) 2 min after carbon. The vein V and its branches appear darkened; there is no sign of vascular labeling due to surgical injury. (2) 2 min, 25 sec after carbon and 15 sec after bradykinin. Numerous annular spasms begin to appear on the larger vein, while the arteriole (arrow) becomes greatly dilated. (3) 55 sec after bradykinin. The spasm of the larger vein has reached its maximum; venular labeling is well under way. The arteriole has become still more prominent. (4) 1 min, 50 sec after bradykinin. The venous spasm is relaxing; strong labeling of the venules.
HISTAMINE-TYPE MEDIATORS

In summary, there is no compelling evidence to support the vaso-spastic theory of histamine-type leakage. It is possible, of course, that venous spasm may play a secondary role. Most physiologic studies already published (6-9) deal with the general effect of histamine administered intravascularly and relatively high doses are necessary to obtain venous constriction (7, 8). The general impression one derives from these experiments is that we are dealing with larger vessels relating to the systemic effect of the drug; these larger vessels are not directly pertinent to the local phenomena of inflammation.

In the single study known to us in which venular calibers were carefully measured (17), three compounds causing vascular leakage were tested on the rat mesocecum. Serotonin caused a significant constriction of "muscular venules" (54.9 ± 2.6 to 43.0 ± 2.0 μ); histamine caused a slight dilatation (47.9 ± 1.2 to 52.8 ± 1.6 μ) and bradykinin a strong dilatation (44.7 ± 1.9 to 61.6 ± 2.4 μ). These data, besides proving once again the different reactivity of different vascular beds, clearly demonstrate that venular leakage occurs independently of venous constriction.

A Conciliatory Hypothesis of Vascular Leakage, as Induced by Histamine-type Mediators

This concept arose from a fortuitous electron microscopic observation suggesting that the endothelial cells have the power to contract, in fact, that they respond to histamine, serotonin and bradykinin by active contraction—at least in the venules. The results have been reported in a preliminary fashion (18). In essence, after application of the mediators in question, we found that many nuclei of endothelial cells appear greatly distorted, recalling the deformation seen in contracted smooth muscle cells. Many cells also bulge into the lumen, with a "hunched" appearance quite unlike that which one would expect in the lining of a distended venule. Thus the endothelial cells (in the venules) appear to respond to certain pharmacologic agents in the same manner as smooth muscle. The suggestion is not far-fetched. There are considerable links between the two types of cells, both structural...
and biological (11). Endothelial cells have long been known to contain very fine fibrils (11). More recently, Cotran has observed (in endothelial cells of vessels in rat skin) larger bundles of fibrils with a periodic transverse striation (19); we have also encountered such structures, and others have described them in the endothelium of uterine arteries in the rat (20). Furthermore, Becker and Murphy have observed that fluorescein-conjugated antibodies to human uterine actomyosin stain endothelial cells of human blood vessels, indicating that they contain actomyosin antigenically similar to uterine actomyosin (personal communication). While the problem requires deeper study, it appears that the endothelium does not lack a morphological substrate for contraction.

In future studies of vasoconstrictor agents on venules it will be important to correlate the changes in caliber of a given vessel with the presence or absence of smooth muscle. In the absence of smooth muscle, contraction of a venule might depend not only on the endothelium but also on the pericytes, which are always present (whether they may also contract we do not know, though we suspect that they do). As to the single venule in our experiments that went into spasm (Fig. 8), we can offer no conclusive explanation for its aberrant behavior. Venules of its caliber, in the rat cremaster, usually have no smooth muscle. The spasm may have been due to endothelial contraction, the unusual effect on the lumen possibly being favored by an unusually low intraluminal pressure.

On the basis of the evidence presented so far, we therefore propose the following sequence of events after application of histamine-type substances. There is an immediate dilatation of the arterioles, which will raise the pressure in both capillaries and venules; the mechanism of this dilatation remains unknown. At the same time, the endothelial cells of the venules respond by contraction; since they contract in a vessel under high pressure, their pulling does not result in a decrease in diameter of the lumen, but in a partial detachment of the intercellular connections. With regard to the venule as a whole, the situation is comparable to that of an isometric contraction. If the larger veins contract this may help to raise the intravenous pressure, as does the arteriolar dilatation; but it is not a necessary event. This sequence of events seems to fit all the known facts. It shows that "contraction" of some sort is indeed involved; it takes into account the importance of hydrostatic pressure, and of venous contraction where and when it occurs; it explains the perplexing fact, noticed by others (14) and illustrated herein, that venules may leak without apparent change in their diameter (passive dilatation would be offset by active endothelial contraction); and above all, it can account, once again, for the fact that the same agents which cause histamine-type leakage are also agents which induce smooth muscle to contract. Further studies are planned to test the viability of this concept.

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References

HISTAMINE-TYPE MEDIATORS

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