The Mechanism of Vascular Leakage Induced by Leukotriene E_4

Endothelial Contraction

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This study identifies the microvascular target of leukotriene E_4 (LTE_4) by vascular labeling with carbon black and establishes the mechanism of its action at the cellular level by electron microscopy. LTE_4 and its tripeptide precursor, leukotriene C_4 (LTC_4) were injected subcutaneously in guinea pigs. With LTE_4, venular labeling was intense at 1000 and 100 ng and slight at 10 ng, with extinction at 1 ng. LTC_4 induced a ring of labeled venules around a blank central area, suggestive of vasospasm. The nonpeptidyl leukotriene LTB_4 induced no labeling. Histamine (1000 ng) induced an area of vascular labeling about equal to that by 1000 ng LTE_4, but the labeling of individual venules was more intense. By electron microscopy, LTE_4 was found to induce gaps in the endothelium of the venules; the endothelial cells adjacent to the gaps bulged into the lumen and showed wrinkled nuclei, consistent with cellular contraction. This ultrastructural evidence suggests that LTE_4 increases vascular permeability by contraction of endothelial cells selectively, in the postcapillary venules, as was previously demonstrated for other inflammatory mediators, including histamine, serotonin, and bradykinin. (Am J Pathol 1987, 126: 19–24)

VASCULAR leakage is a cardinal effect of certain inflammatory mediators, and it is well established that it occurs almost exclusively in the postcapillary (pericytic) venules[1] with a diameter in the range of 10–50 μ. At the cellular level, the mechanism of this leakage was demonstrated almost two decades ago for histamine, serotonin, and bradykinin by electron-microscopic studies, which were confirmed *in vivo*[2,3]: endothelial cells in the postcapillary venules contract, and thereby pull away from each other. With the more recent description of many additional endogenous permeability-increasing agents, it seemed possible that some might act on a microvascular target other than the venules and/or by a mechanism other than cellular contraction. For a few of the more recently described lipid mediators, including leukotriene C_4 (LTC_4), leukotriene D_4 (LTD_4), and the platelet activating factor acetyl glyceryl ether phosphorylcholine, the postcapillary venules are once again the selective targets of increased permeability,[4,5] but the cellular mechanism has not been addressed by morphologic studies.

Leukotriene E_4 (LTE_4), like its metabolic precursors LTC_4 and LTD_4 (Figure 1), is a component of the "slow reacting substance of anaphylaxis" (SRS-A), which was initially described as a product of an immediate-type hypersensitivity reaction, but subsequently suggested to be produced in response to a broader variety of inflammatory stimuli.[6,7]

Since LTE_4 increases vascular permeability in the guinea pig skin[8] and trachea,[9] hamster buccal mucosa,[4] and human skin,[10] and, unlike LTC_4 and LTD_4, it does not induce cutaneous arterial constriction in the guinea pig,[8] it was selected for emphasis in the present study. This was particularly necessary because arterial constriction would tend to decrease vascular leakage and therefore mask a permeability-increasing effect. The emphasis on LTE_4 among the SRS-A components affecting vasopermeability was

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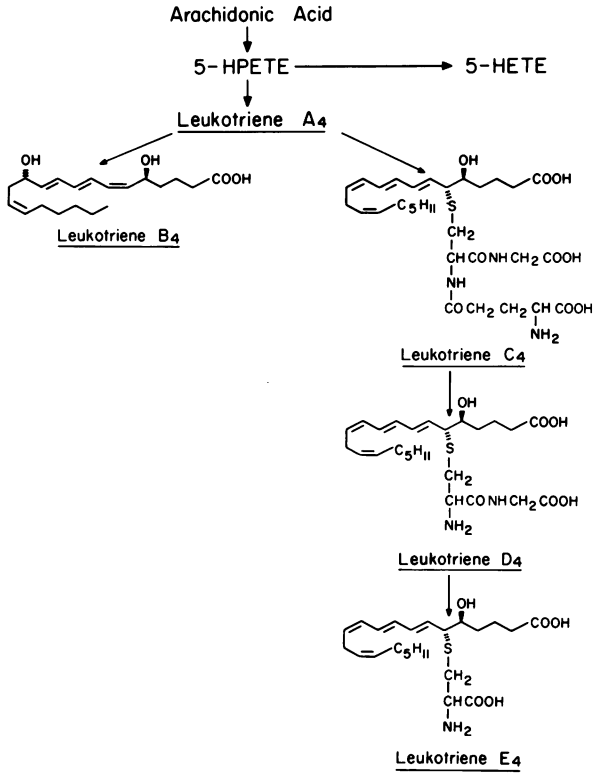

Figure 1 — The 5-lipoxygenase pathway with chemical structures of the leukotriene products.

also appropriate because intravascular metabolism of LTC_4 and LTD_4 to LTE_4 occurs readily in plasma,[11,12] and, of the three, only LTE_4 can be recovered as a stable urinary product after intravenous injection of LTC_4 in man.[13]

Materials and Methods

LTB_4, LTC_4, LTE_4 were each prepared by total organic synthesis as previously described[14], and each was resolved as a pure compound by reverse phase-high performance liquid chromatography just before use in the biologic experiments. Histamine diphosphate was purchased from Sigma Chemical Company (St. Louis, Mo).

Thirty-three Hartley strain guinea pigs, 300–500 g each, were used. Under ether anesthesia the abdominal skin was shaved and the test substance was injected subcutaneously in doses ranging from 1 to 1000 ng in 100 μl saline. Each animal received four injections of one test substance at least 50 mm apart. Immediately thereafter, colloidal carbon black (Pelikan Biological Ink, Batch C11/1431a, Gunther Wagner, Hanover, West Germany) was injected into the brachial vein in doses of 0.1 ml/100 g body weight for the purpose of labeling all hyperpermeable vessels.[1] The effect of pretreatment with a cyclooxygenase inhibitor was tested in 3 guinea pigs: 30 minutes prior to the subcutaneous injections of LTE_4 these animals received 30 mg/kg of indomethacin intraperitoneally. All guinea pigs were euthanized with ether, either 4–15 minutes after the carbon injection (for electron-microscopic studies) or 30–60 minutes after the same injection (for light microscopic studies). The abdominal skin, its dissected intrinsic muscle, and the abdominal muscles were excised; either they were fixed in formalin, cleared in glycerin, and examined by transillumination for a topographic study of the vessels, or they were fixed in 3% glutaraldehyde, dehydrated, embedded in Epon 812, and examined by electron microscopy.

Results

By light microscopy LTE_4 was found to induce diffuse and extensive postcapillary venular labeling at 1000 and 100 ng and slight labeling at 10 ng, with extinction at 1 ng. At the highest dose, the labeled area of abdominal muscle in different animals had diameters of up to 4.5 cm; the individual venules were labeled rather faintly, but distinctly (Figure 2). The leaky (labeled) vessels were postcapillary (pericytic) venules; no capillaries and no arterioles were involved (Figure 3a). With histamine, the same overall pattern of labeling was seen but the individual postcapillary venules appeared to be labeled more heavily (Figure 3b). One thousand nanograms of LTC_4 produced either no labeling at all (2 animals) or a ring of labeling around a nonlabeled area (1 animal); 100 and 10 ng of LTC_4 produced a narrow zone of faint labeling

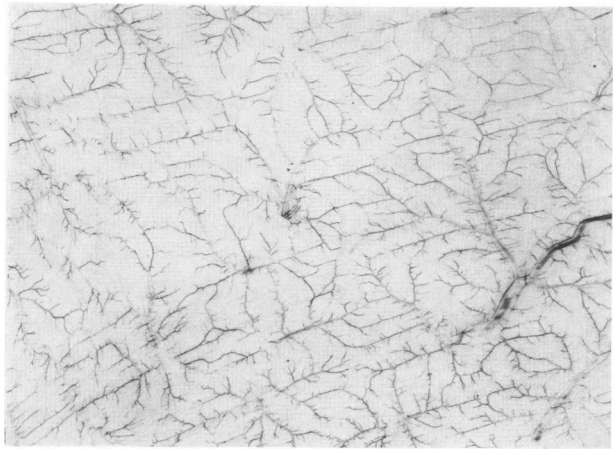

Figure 2 — Venular labeling with carbon black in the abdominal muscle, after local subcutaneous injection of LTE_4 (1000 ng). (×2.6)

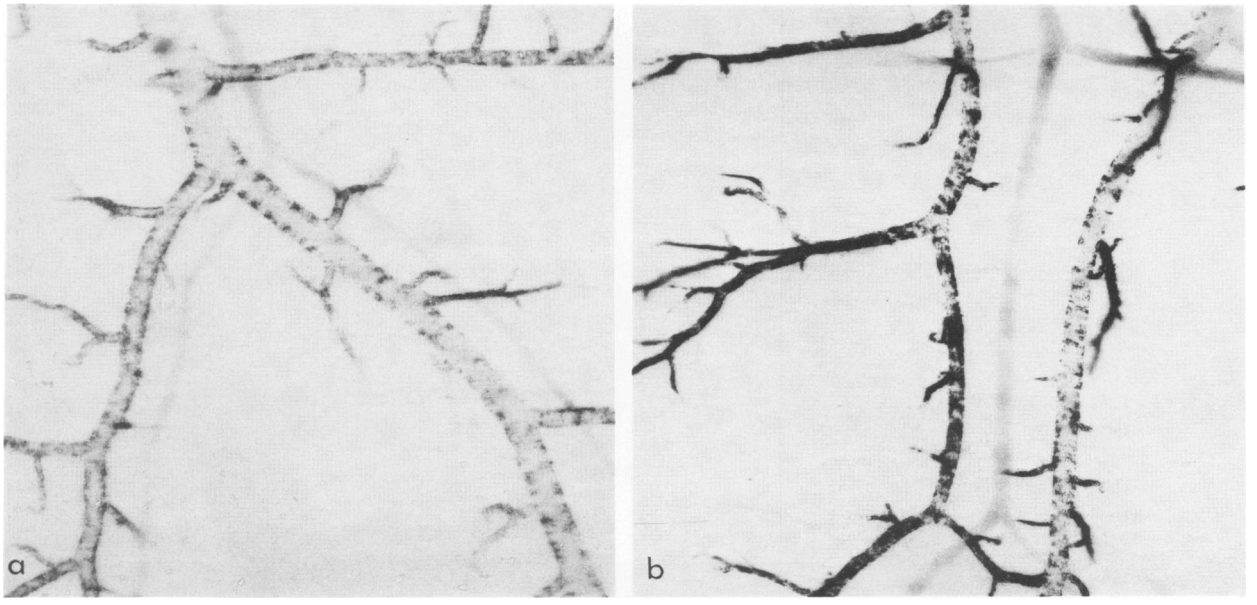

Figure 3—Venular labeling with carbon black in the abdominal muscle. Note that arterioles and capillaries are not labeled. (×33) **a**—LTE_4 (1000 ng). **b**—Histamine (1000 ng). The labeling is more intense with histamine.

around a circular unlabeled area; 1 ng produced faint traces of labeling. LTB_4 up to 1000 ng did not induce labeling. In the guinea pigs pretreated with indomethacin, carbon labeling induced by LTE_4 was the same as in controls (animals injected with LTE_4 but without pretreatment). By electron microscopy, the postcapillary venules of the skin and muscles exhibited intercellular gaps filled with carbon and occasional platelets after LTE_4 injection. The adjacent endothelial cells were bulging, their nuclei were rounded, and their membranes showed many infoldings (Figure 4a); whereas in controls the endothelial cells were elongated and flattened, the nuclei had a smooth outline, and no gaps were seen (Figure 4b).

Discussion

At the microvascular level, the patterns of labeling obtained with LTE_4 and LTC_4 were the same: postcapillary (pericytic) venules only were affected, as is the case with histamine, serotonin, bradykinin[1] and LTD_4[4]. For LTC_4 this result was anticipated, because studies on the cheek pouch *in vivo* have shown that leakage of fluorescent probes occurs in this segment of the vascular tree.[4] The selection of the venules as vascular targets for histamine is apparently due to a localized concentration of histamine (H-1) receptors.[15] Data on the distribution of (SRS-A) leukotriene receptors on vascular endothelium is still scanty and limited to receptors for LTC_4 on aortic endothelium *in vitro*.[16]

Topographically, there was a major difference between the effects of LTE_4 and LTC_4: LTE_4 labeled all the postcapillary venules within a roughly circular area (Figure 2); in contrast, LTC_4 induced a ring-shaped area of venular labeling around a pale center, which was not only free of labeled venules but also of visible blood vessels. We interpret these results to indicate that LTC_4 induces both increased permeability and arterial spasm.[17] In the center, the spasm would predominate and hence give the appearance of avascularity; the lack of flow precludes labeling any leaky vessel. At the periphery some flow would be preserved, perhaps by dilution of the mediator and/or by anastomoses with surrounding vessels, and venular labeling can occur.

Despite the different effects of LTE_4 and LTC_4, it could be argued that the permeability-increasing effects observed were not due directly to the injected leukotriene (a product of the lipoxygenase pathway) but indirectly, to some product of the cyclooxygenase pathway. Therefore, we tested the effect of LTE_4 on guinea pigs pretreated with an inhibitor of the cyclooxygenase pathway, indomethacin: there was no difference in the labeling pattern, indicating that the observed permeability effects were indeed due directly to LTE_4.

There was an interesting difference between the LTE_4 and the histamine-stimulated labeling of venules, in that venules labeled after histamine injection were darker (Figure 3a and b). Although the gaps induced by histamine could be larger and therefore

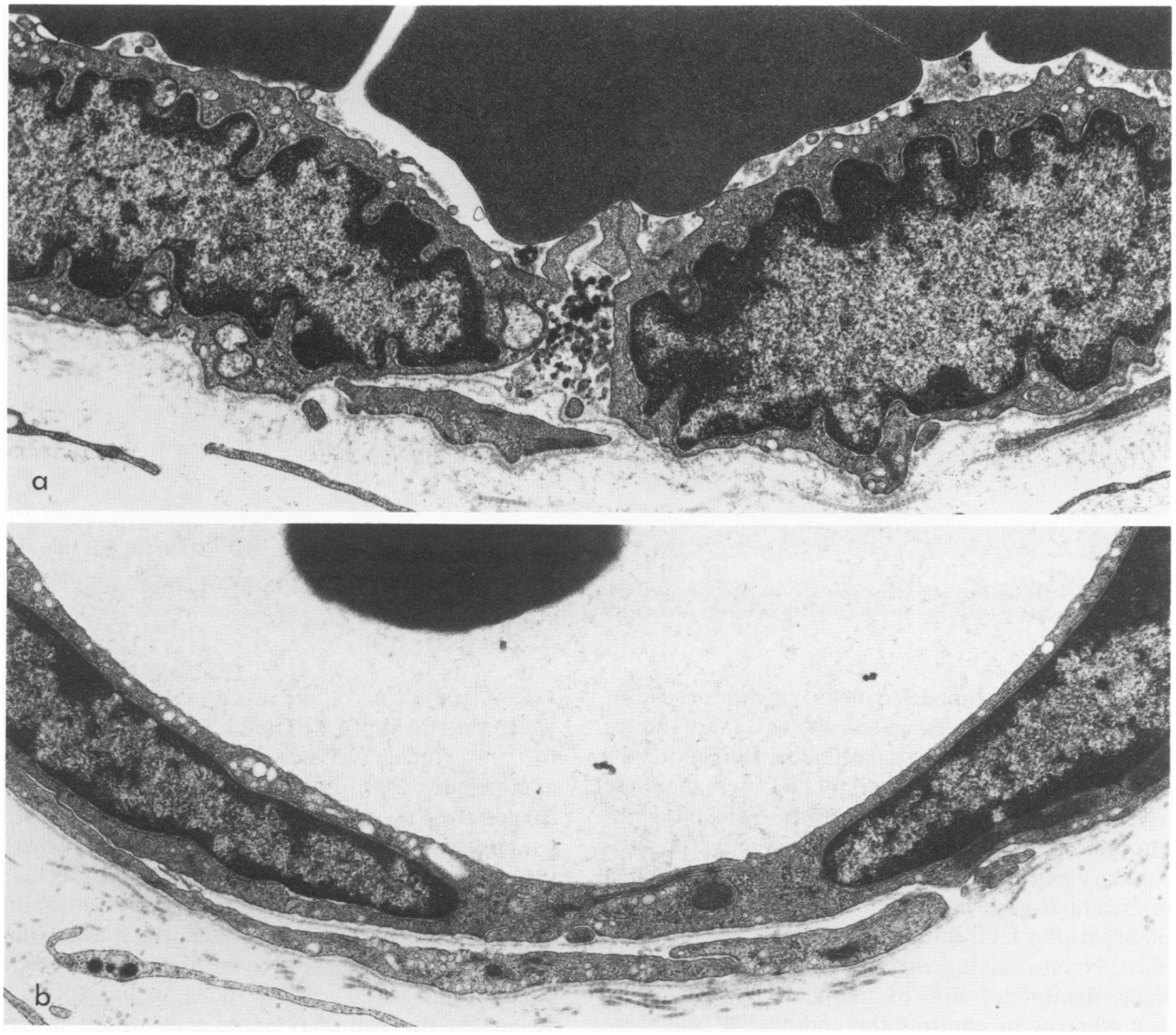

Figure 4a — Venule in the abdominal muscle 11 minutes after intradermal injection of LTE_4 (830 ng) and intravenous injection of carbon black. The endothelial cells bulge into the lumen; their nuclei show many indentations. The gap between the endothelial cells is filled with colloidal carbon. Packed red blood cells in the lumen indicate plasma leakage. (×18,200) **b** — Control venule (abdominal muscle). The endothelial cells are elongated, flattened, and connected by a tight junction; their nuclei have a smooth outline. (×14,200)

cause more leakage, this is not supported by ultrastructural evidence. We would propose a more likely alternative mechanism: since the amount of colloidal carbon deposited in the venular wall should be proportional to the amount of plasma seeping through the gaps, and thus to the blood flow, the histamine effect might be enhanced due to hyperemia, whereas that of LTE_4 might be limited by an unchanged or reduced blood flow.

The electron-microscopic findings were practically identical to those described earlier for histamine, serotonin, and bradykinin[2]: the endothelium of capillaries and arterioles remained unchanged, whereas the postcapillary venules showed intercellular leaks; additionally, the endothelial cells adjacent to the leaks were bulging, with rounded nuclei characterized by many infoldings of the nuclear envelope (Figure 4a and b). We conclude that LTE_4 induces venular leakage by the same mechanism proposed for the above-named mediators, namely, endothelial contraction. Although the evidence is from ultrastructure alone, there seems to be no other reasonable way to interpret the cellular changes observed.

The concept of endothelial cell contraction as an effect of inflammatory mediators is now generally accepted. Besides ultrastructural evidence, it is supported by many other data: the phenomenon has been observed *in vivo*[3] as well as in cultured endothelial

cells exposed to histamine or thrombin[18-20]; it has also been confirmed by quantitative studies *in vitro*.[21] Most significantly, direct evidence of endothelial contraction has been provided by demonstrating that cultured endothelial cells, applied to a platelet-free fibrin network, are able to retract it if stimulated by thrombin.[20] Moreover, venular leakage can be inhibited with cytochalasin B and by EDTA/EGTA[22]; it is generally caused by agents that induce smooth-muscle contraction and inhibited by agents with relaxant properties (β-adrenergic agonists, calcium antagonists).[23] Since the limited objections to this view derive from a single source,[24-28] its arguments should be examined in some detail. The alternative view proposes that the endothelium becomes leaky because of "increased membrane motility".[28] This notion is based on electron micrographs of ischemic tissues and experimental melanomas, in which blood vessels show gross endothelial distortion and membrane damage, such as blebbing and myelin figures. There is no reason to assume that these models relate to the effects of mediators in inflammation. The suggestion has also been made on the basis of the same models, that endothelial cells separate along their junctions because their membranes form projections like teeth of a "defective zipper," forcing the cells apart. This hypothesis is inconsistent with published experiments from as early as 1969 which show that after histamine injection, endothelial cells retract while maintaining taut filamentous bridges that are capable of bisecting an escaping erythrocyte.[29] Serial sections and computerized three-dimensional reconstructions of gaps induced by histamine have confirmed that the retracted cells remain focally connected by cytoplasmic processes.[30] Such processes are also strikingly demonstrated in scanning electron micrographs of endothelial cells contracting in tissue culture.[31] Overall, these arguments against endothelial contraction fail to distinguish between the *physiologic cellular response* of contraction induced by inflammatory mediators and a *nonspecific cellular pathology* as is found in toxic, osmotic, and ischemic injury or in neoplastic growth.

Of course, other mechanisms may exist for causing endothelial cells to separate. The catalytic actions of collagenase and trypsin as used routinely for the isolation of endothelial cells could hypothetically be a model for an *in vivo* enzyme-mediated event. Also, it has long been known that *direct injury* by mild heat or toxic agents can cause endothelial junctions to open without evidence of cellular contraction.[32-34] One toxic agent, ethchlorvynol, induces pulmonary edema *in vivo* and intercellular gaps between endothelial cells in tissue culture; in this case, gap formation may be caused by focal rearrangements of the cytoskeleton,[31] as shown by directly exposing lung microvasculature to cytochalasin B or D.[35]

Last, a role for the pericytes must be considered. There is morphologic evidence for their contractility,[36] and it is now established that they contain protein components of a contractile system.[37-40] Because of their position in the venular wall, it is not clear how they could bring about leakage; however, they may control fluid loss by affecting the diameter and/or the tone of the venules.

In summary, we have provided evidence that a recently described permeability-inducing agent, LTE$_4$, causes postcapillary venules to leak by the same mechanism as shown for the action of histamine, serotonin, and bradykinin: endothelial contraction. The single published electron micrograph of a venule leaking under the effect of platelet-activating factor (PAF) shows endothelial changes suggestive of contraction.[5] It would be interesting to know whether this mechanism applies generally to all permeability-increasing inflammatory mediators, now known as "autacoids."

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