Leukotrienes promote plasma leakage and leukocyte adhesion in postcapillary venules: *In vivo* effects with relevance to the acute inflammatory response

(hamster cheek pouch/microcirculation/icosanoids/slow reacting substance of anaphylaxis/histamine)

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ABSTRACT   Leukotrienes B$_4$, C$_4$, and D$_4$, members of a recently discovered family of substances biosynthesized from arachidonic acid, were found to have potent microvascular actions in the hamster cheek pouch. When applied topically to the vascular network, leukotrienes C$_4$ and D$_4$ caused an intense constriction of arterioles, being similar to angiotensin in potency in this respect. The vasoconstriction induced by leukotrienes C$_4$ and D$_4$ was short-lived, and it was consistently followed by a marked and dose-dependent extravasation of macromolecules from postcapillary venules. Histamine did not constrict arterioles, but it elicited leakage of plasma, although on a molar basis it was no more than 1/1000th as potent as the leukotrienes. When used in the same concentration range as leukotrienes C$_4$ and D$_4$, leukotriene B$_4$ did not evoke vasoconstriction or promote plasma leakage. On the other hand, leukotriene B$_4$ caused a conspicuous and reversible adhesion of leukocytes to the endothelium in postcapillary venules. Our findings that leukotrienes induce microcirculatory alterations *in vivo*, closely resembling the early events in the acute inflammatory response, imply that leukotrienes, formed in several blood-borne and tissue-bound cells, may mediate important microcirculatory adjustments to noxious stimuli.

The leukotrienes are a newly discovered family of bioactive substances derived from polyunsaturated fatty acids (e.g., arachidonic acid) (Fig. 1) (1–4). Leukotriene biosynthesis is initiated by a lipoxygenase catalyzing the formation of a 5-hydroperoxy acid, which then may form leukotriene A(LTA), the unstable epoxide intermediate having the characteristic conjugated triene structure present in all leukotrienes. LTA may add water to form leukotriene B (LTB), or it may add gluthathione to yield leukotriene C(LTC). Successive enzymatic removals of glutamic acid by γ-glutamyl transpeptidase and of glycine by a dipeptidase convert LTC into its corresponding cysteinylglycyl and cysteinyl analogues, LTD and LTE, respectively.

It is now evident that leukotrienes comprise the intriguing entity slow reacting substance of anaphylaxis (SRS-A) (1–4), a proposed mediator of hypersensitivity reactions (5). This was first demonstrated by using a SRS generated from murine mastocytoma cells (6). Pharmacological analysis confirmed that LTC$_4$ and LTD$_4$ have a spectrum of biological actions closely similar to that previously reported for SRS-A (7, 8). It has been shown that both immunologically and nonimmunologically generated SRS-A can be identified as an entity consisting of several closely related leukotrienes, principally the cysteinyl leukotrienes of the C, D, and E series (9–17).

During investigations of the biological activity profile of leukotrienes by using a standard assay for SRS-A, the guinea pig
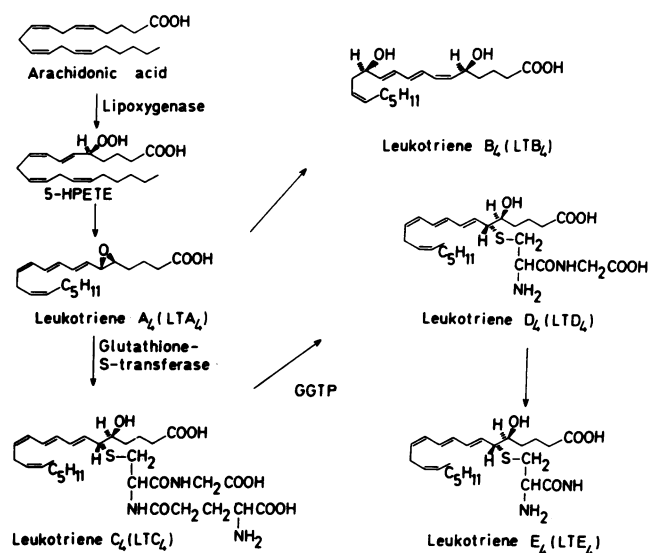

FIG. 1. Biosynthetic pathway for leukotrienes generated from arachidonic acid; subscript 4 denotes the presence of four double bonds. Leukotrienes with three or five double bonds can be derived from 5,8,11- or 5,8,11,14,17-icosaenoic acids. For details on nomenclature, see ref. 4. GGTP, glutamyl transpeptidase.

skin test (5), intracutaneous injections of LTC$_4$ and LTD$_4$ were found to cause a marked and dose-dependent extravasation of Evans blue (7). As also reported by others (8), we noted that LTD$_4$ produced a homogeneous blue spot, whereas spots obtained by equivalent doses of LTC$_4$ often had a central blanching, similar to what has been reported for high doses of other agents that increase vascular permeability (18).

Because the reason for this difference was not obvious, it was considered of interest to analyze further the microvascular effects of leukotrienes. The limitations of the skin test made us turn to the hamster cheek pouch preparation, in which the terminal vascular bed can be studied under *in vivo* conditions (19, 20). The experimental design permits simultaneous observations on the extent and localization of different vascular events, such as blood flow, vessel diameter, macromolecular permeability, and blood cell–endothelium interaction (21, 22).

Here, we report that LTC$_4$ and LTD$_4$ have potent effects on vascular caliber and permeability, and that LTB$_4$ increases leu-

Abbreviations: LTA$_4$, leukotriene A$_4$; LTB$_4$, leukotriene B$_4$; LTC$_4$, leukotriene C$_4$; LTD$_4$, leukotriene D$_4$; SRS-A, slow reacting substance of anaphylaxis; FITC-dextran, fluorescein isothiocyanate-conjugated dextran.
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kocyte adhesion to the endothelium. These findings indicate that, in addition to the effects previously observed with extracts of SRS-A, leukotrienes have actions that imply a role in acute inflammatory reactions.

MATERIALS AND METHODS

Male golden hamsters (*Mesocricetus auratus*) weighing 60–110 g were anesthetized with sodium pentobarbital (60 mg/kg, intraperitoneally). Supplemental doses of the anesthetic were given through a fine polyethylene catheter introduced into the femoral vein. The animals were allowed to breathe spontaneously through a tracheal cannula, and the body temperature was maintained at 37°C.

The cheek pouch was prepared for intravital microscopy of macromolecular permeability according to Svensjö *et al.* (21). Briefly, the cheek pouch was gently everted, and the distal, nonmuscular part was mounted on a ring of silicone rubber in a transparent circular well. After incision of the superficial layer of the pouch, the underlying connective tissue was carefully dissected away, exposing the vascular network in the bottom layer (thickness, 100–125 μm) for observation in the microscope (Leitz Ortholux). During the preparation and throughout the experiment the pouch was superfused (6 ml/min) with a salt solution (composition in mM: NaCl, 131.9; KCl, 4.7; CaCl, 2.0; MgSO$_4$, 1.2; NaHCO$_3$, 18.0) maintained at 37°C and gassed with 5% CO$_2$ in N$_2$ to give a pH of 7.35 and a pO$_2$ of approximately 3 kPa.

In studies on vascular permeability, the microscope was equipped with ×3.5 objectives and ×12.5 oculars (magnification approximately ×44) and proper filters for fluorescein excitation and emission. The preparation was transilluminated with a 200-W Hg lamp (Leitz, 301-179-200). Fifteen minutes after completion of the preparation, fluorescein isothiocyanate-conjugated dextran (FITC-dextran, M_r 150,000) was given intravenously (2.5 mg/kg) to act as a tracer of macromoleuclar leakage. The number of leakage sites having a diameter >100 μm can be used as an index of macromolecular extravasation (21). The number of such fluorescent spots per cm^2 was determined at 5-min intervals throughout the experiment, starting with a control period of 20 min. Preparations showing more than 10 spots per cm^2 during this control period were discarded. Thereafter, the superfusion was stopped and the substance to be tested was added to the bath solution. Three minutes later the drug was washed away as the superfusion was begun again, and the number of leakage sites was determined for another 20 min or more.

In studies on leukocyte behavior, the microscope was equipped with a ×55 water-immersion objective and ×12.5 oculars (magnification, approximately ×690). The light source was a 100-W Hg lamp (IREM, EI-XH5 P/L). After an equilibration period of 30 min, a postcapillary venule (diameter, 10–16 μm) was chosen and the image was projected on a TV monitor via a camera (National WV-1050 E/C) and stored on videotape. Two lines were drawn on the TV screen, perpendicular to the vessel, enclosing a distance of 100 μm. In the observed 100-μm segment of the vessel, the leukocytes moving in the periphery of the axial stream were considered to be "rollers," and those adhering to the endothelium and remaining in the same position for 1 min were considered to be "stickers" (22). The numbers of rolling and sticking leukocytes were determined in duplicate determinations on three to five occasions in three successive 10-min periods. During the second period, LTB$_4$ was infused into the medium continuously superfusing the cheek pouch. Erythrocyte velocity and the diameter of the vessel were used as indices of the rate of blood flow. Erythrocyte velocity was determined according to the dual-slit cross-cor-

relation technique (23), and the diameter of the vessel was measured on the TV screen.

LTB$_4$, LTC$_4$, and LTD$_4$ were generated biosynthetically, as described (6, 9, 24, 25), and stored in methanol or ethanol at −25°C. Dilutions were made in the superfusion medium immediately before use; the final concentration of ethanol, not

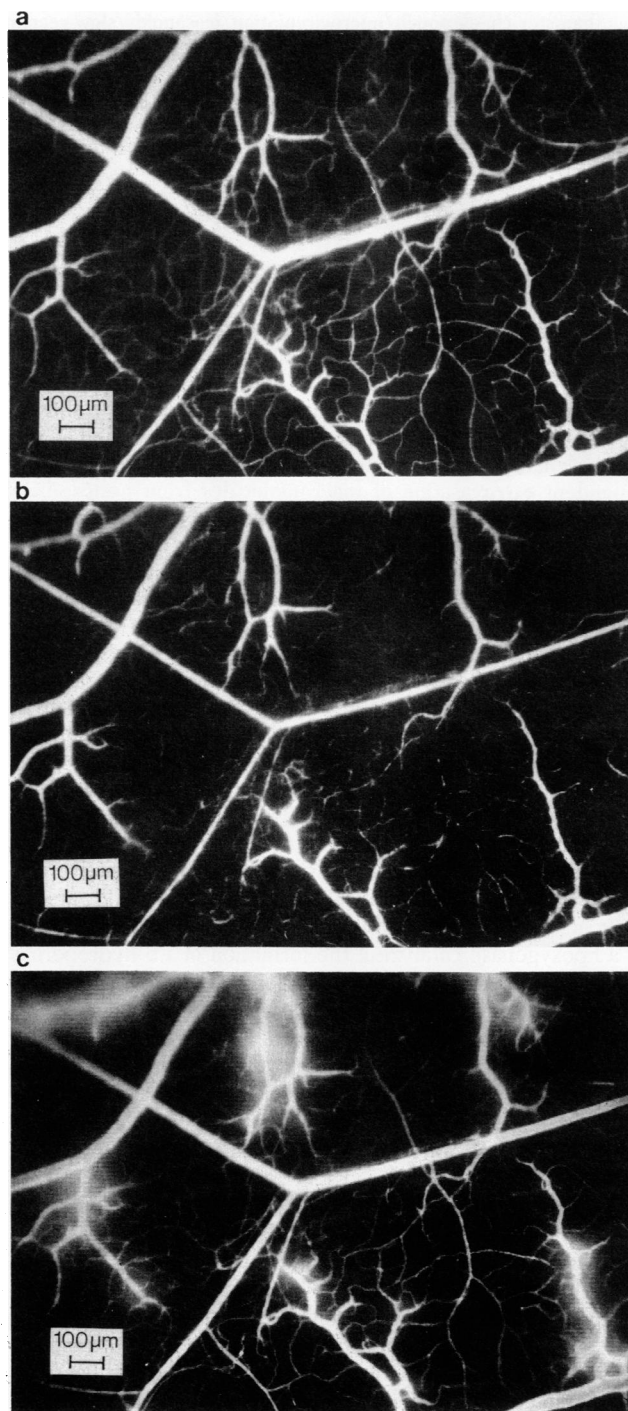FIG. 2. Cheek pouch microvasculature at ×44 (bar denotes 100 μm). Exposure time, 10 sec. (a) Straight Y-shaped vessel traversing view is an arteriole; the more tortuous vessels are venules. (b) At 1 min after application of 4 nM LTD$_4$. Note pronounced arteriolar constriction, narrowed Y-shaped arteriole, and apparent disappearance of several terminal arterioles (e.g., the arteriole running vertically in the center of the field in a). (c) At 5 min. Vasoconstriction has ceased, but now vascular permeability is increased, as indicated by leakage of FITC-dextran from postcapillary venules.

exceeding 0.02%, had no effect on extravasation of FITC-dextran or on leukocyte adhesion. Histamine was obtained from Sigma; angiotensin II was from CIBA–Geigy. All other chemicals were of reagent grade.

Statistical hypotheses were tested by Student's *t* test for paired and unpaired variates.

RESULTS

The immediate response to LTC_4 and LTD_4 (0.3–20 nM) was a prompt and dose-dependent constriction of arterioles (Fig. 2 *a* and *b*). Terminal arterioles were exquisitely sensitive, often closing totally and opening again only when constriction of larger arterioles vanished. LTC_4 and LTD_4 seemed to be equipotent in this respect. Furthermore, both leukotrienes were effective vasoconstrictors in the same low dose range as angiotensin (0.3–3 nM). When arteriolar constriction induced by LTC_4 and LTD_4 ceased, there consistently was a dose-dependent extravasation of FITC-dextran (Fig. 2*c*). Angiotensin, on the other hand, did not increase plasma leakage, indicating that even intense vasoconstriction *per se* does not necessarily induce increased vascular permeability.

The vascular effects of the leukotrienes were also compared with those of histamine which is considered to be an important mediator of vascular events in inflammatory reactions. In agreement with previous findings in the hamster cheek pouch, histamine induced a marked and dose-dependent increase in vascular permeability (26). However, histamine did not cause arteriolar constriction, which might serve to explain why the peak of histamine-induced leakage appeared earlier than did leakage induced by leukotrienes. The duration of plasma leakage, however, was virtually the same for leukotrienes and histamine, but this could be due to the effective washing away of applied substances in the present experimental design rather than to similarities in duration of action for histamine and leukotrienes. Notably, plasma leakage induced by histamine and leukotrienes was confined to postcapillary venules, in accordance with the concept that this vessel segment is the specific target of action for agents inducing reversible changes in vascular permeability (27).

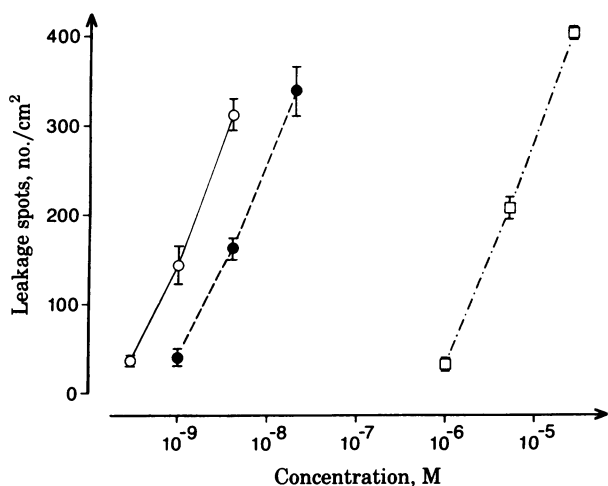The potency of histamine and leukotrienes in causing in-

creased vascular permeability was established from single observations in each pouch, because of a clear-cut tachyphylaxis to repeated administration of leukotrienes, similar to that observed in various airway preparations (7, 28). Dose–response curves so obtained were parallel for histamine, LTC_4, and LTD_4, and maximal responses of similar magnitude could be elicited with histamine and leukotrienes (Fig. 3). On a molar basis, however, LTC_4 and LTD_4 induced a significant increment of vascular permeability at much lower concentrations than did histamine. LTC_4 was most effective in this respect, being approximately 5000 times more potent than histamine, and about 5 times more potent than LTD_4.

LTB_4, in the same dose range as LTC_4 and LTD_4, had no effect on vascular caliber and permeability but, in contrast to LTC_4 and LTD_4, it caused leukocytes moving in the periphery of the axial stream to adhere to the vessel wall. Although this phenomenon was seen in venules of various caliber, it was found to be especially prominent in postcapillary venules of diameter <15 μm (Fig. 4). This vessel segment was chosen for further observations on leukocyte–endothelium interactions, in partic-

FIG. 3. Dose-dependent increase in vascular permeability of the hamster cheek pouch caused by LTC_4 (o——o), LTD_4 (●– – –●), and histamine (□·····□). The number of FITC-dextran leμm in diameter were counted at 5-min intervals and used as an index of macromolecular extravasation (21). Each value represents mean (±SEM) of maximal leakage obtained from five separate experiments for each dose of agonist.
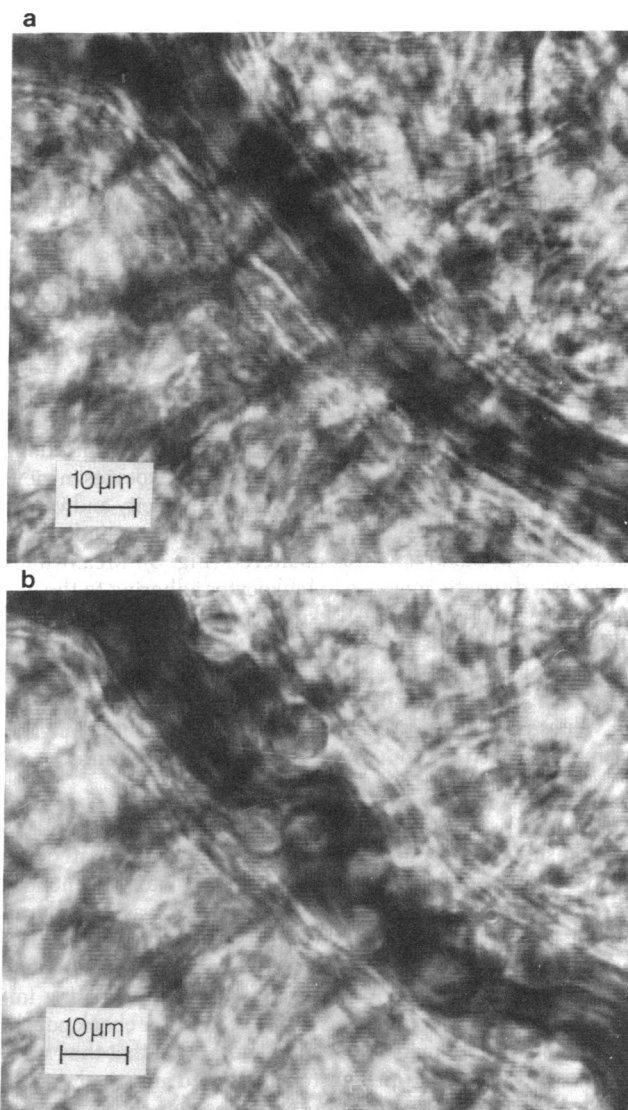

FIG. 4. Postcapillary venule in the hamster cheek pouch viewed at ×690 through a water-immersion objective, (bar represents 10 μm). Exposure time, 0.1 sec. (*a*) Control. (*b*) LTB_4 (final concentration, 4 nM) present for 5 min in medium continuously superfusing the pouch. Note the great number of leukocytes adhering to the vessel wall.
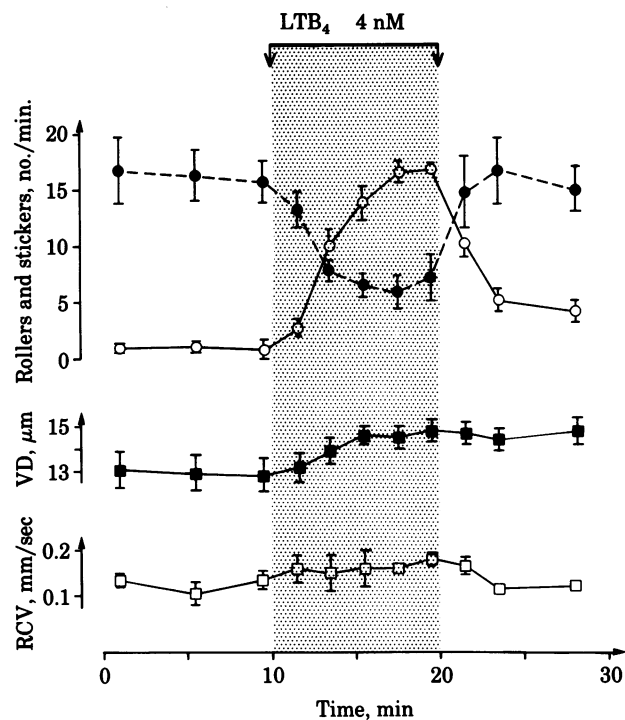
FIG. 5. The number of leukocytes rolling (●--●) and sticking (○—○) to the vessel wall in a postcapillary venule of the hamster cheek pouch, counted during three successive 10-min periods, as described in text. LTB$_4$ added to the superfusion medium (final concentration, 4 nM) caused a decrease in the number of rollers and a marked increase in the numbers of stickers. Simultaneous measurements were made of vessel diameter (VD) and erythrocyte velocity (RCV). Values are expressed as mean (±SEM) of duplicate determinations.

ular because it is considered to be the major site for leukocyte adhesion to the vascular endothelium in response to noxious stimuli (29). Shortly after addition of LTB$_4$ (final concentration, 4 nM) to the medium superfusing the pouch, leukocytes in view began to move at a slower rate. Erythrocyte velocity and vessel diameter were not significantly changed, however, indicating that the rate of blood flow was unaltered (Fig. 5). Meanwhile, there was a dramatic increase in leukocytes adhering to the endothelium, parallelled by a marked decrease in the number of rolling cells. This effect was visible within 1 min, reached its maximum after 6–8 min, and remained maximal until LTB$_4$ was withdrawn. The number of rollers then rapidly returned to control values, while the stickers disappeared more gradually, to reach the values preceding infusion within 10–15 min.

DISCUSSION

The hamster cheek pouch has proved to be a sophisticated and highly reliable model for studying microcirculatory events under *in vivo* conditions (19–22, 26). Hence, it was considered suitable for further analysis of the edema-forming effect of LTC$_4$ and LTD$_4$, previously observed in the guinea pig (7, 8). The leukotrienes proved to be remarkably potent in affecting important microvascular mechanisms in the hamster cheek pouch, even though preliminary experiments indicated that they were considerably less active on hamster airway and intestinal smooth muscle than on corresponding preparations from the guinea pig.

LTC$_4$ and LTD$_4$ were both at least as potent vasoconstrictors as angiotensin and were >1000 times more active than histamine in promoting plasma leakage. Because leukotrienes are generated from leukocytes (3, 9, 12, 24), monocytes (10, 11, 13), macrophages (30), and possibly other cells of the mononuclear

phagocyte system, it is likely that the microvascular bed may be exposed to locally formed leukotrienes. Independently of whether release of leukotrienes is evoked by immunological mechanisms or by other stimuli, the nature and the localization of the vascular effects of LTC$_4$ and LTD$_4$ closely resemble the early response to tissue injury: a short-lived contraction of arterioles, followed by hyperemia and plasma leakage from postcapillary venules (31).

The finding that LTB$_4$ increased leukocyte adhesion to the vessel wall is also of interest in this context because the initial vascular events in inflammation are accompanied by an increased leukocyte adhesion to the endothelium of small venules (29, 31). The observed effect of LTB$_4$ is not likely to be secondary to hemodynamic changes because erythrocyte velocity and vessel diameter were virtually unchanged. If anything, there was a tendency toward increased erythrocyte velocity, which would be expected to decrease leukocyte adhesion to the vessel wall. LTB$_4$ has recently been reported to be a potent chemotactic factor for polymorphonuclear leukocytes *in vitro* (32–35), and the present findings seem to indicate such an effect also under *in vivo* conditions. It has been reported that chemoattractants for leukocytes increase leukocyte adherence to the vascular endothelium (26, 36), but whether this effect is due to changes in leukocyte or endothelium function is not known (29). Regardless of the mechanism for leukocyte adhesion to the endothelium, and irrespectively of whether all types of leukocytes are affected, it is well known that sticking of leukocytes to the vessel wall precedes their emigration from the bloodstream (29, 37).

The microvascular effects of LTC$_4$, LTD$_4$, and LTB$_4$ resemble the structure–activity relationships for leukotrienes also observed in other tissues (7, 28). In the most sensitive airway preparations (e.g., parenchymal strips from guinea pig lungs), all of the cysteinyl leukotrienes (LTC, LTD, and LTE) have the same high bronchoconstrictor potency, whether containing three, four, or five double bonds, or differing in stereochemistry of the C-11 double bond, whereas LTA$_4$ and LTB$_4$ are much less potent (unpublished data). In the present study, LTB$_4$ was much less effective than LTC$_4$ and LTD$_4$ in promoting macromolecular leakage, which might relate to the view that contraction of endothelial cells is a prerequisite for increased vascular permeability in postcapillary venules (38). Therefore, it seems that the cysteinyl substituent at C-6 is of prime importance for the contractile potency of leukotrienes in several systems, whereas other functional groups may be important for effects of LTB$_4$ such as chemotaxis and the increased leukocyte adhesion reported here.

Leukotrienes have been suggested to play a mediator role in immediate hypersensitivity reactions, principally because of their outstanding potency as bronchoconstrictors in man (28) and guinea pig (7, 8, 39). The present finding that LTC$_4$ and LTD$_4$ promote macromolecular extravasation, being at least 1000 times more potent that histamine in this respect, provides additional support for such a view. However, LTC$_4$ and LTD$_4$ were also found to evoke a marked, albeit transient, vasoconstriction, and LTB$_4$ caused increased leukocyte adhesion to the vessel wall, implying that leukotrienes may be involved in vascular events of more general importance. Because leukotrienes are formed in various blood-borne and tissue-bound elements, it may be more than incidental that the triad of effects induced by LTB$_4$ together with LTC$_4$ or LTD$_4$—arteriolar constriction, plasma leakage, and leukocyte adhesion—bears a close resemblance to the early events in acute inflammatory reactions.

1. Samuelsson, B., Borgeat, P., Hammarström, S. & Murphy, R. C. (1979) *Prostaglandins* **17**, 785–787.
2. Samuelsson, B., Borgeat, P., Hammarström, S. & Murphy, R. C. (1980) in *Advances in Prostaglandin and Thromboxane Research*, eds. Samuelsson, B., Ramwell, P. W. & Paoletti, R. (Raven, New York), Vol. 6, pp. 1–18.
3. Samuelsson, B., Hammarström, S., Murphy, R. C. & Borgeat, P. (1980) *Allergy* **35**, 375–381.
4. Samuelsson, B. & Hammarström, S. (1980) *Prostaglandins* **19**, 645–648.
5. Orange, R. P. & Austen, F. K. (1969) *Adv. Immunol.* **10**, 105–144.
6. Murphy, R. C., Hammarström, S. & Samuelsson, B. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4275–4279.
7. Hedqvist, P., Dahlén, S.-E., Gustafsson, L., Hammarström, S. & Samuelsson, B. (1980) *Acta Physiol. Scand.* **110**, 331–333.
8. Drazen, J. M., Austen, F. K., Lewis, R. A., Clark, D. A., Goto, G., Marfat, A. & Corey, E. J. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 4354–4358.
9. Örning, L., Hammarström, S. & Samuelsson, B. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 2014–2017.
10. Bach, M. K., Brashler, J. R., Hammarström, S. & Samuelsson, B. (1980) *J. Immunol.* **125**, 115–118.
11. Bach, M. K., Brashler, J. R., Hammarström, S. & Samuelsson, B. (1980) *Biochem. Biophys. Res. Commun.* **93**, 1121–1126.
12. Morris, H. R., Taylor, G. W., Piper, P. J., Samhoun, M. N. & Tippins, J. R. (1980) *Prostaglandins* **19**, 185–201.
13. Lewis, R. A., Austen, F. K., Drazen, J. M., Clark, D. A., Marfat, A. & Corey, E. J. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 3710–3714.
14. Morris, H. R., Taylor, G. W., Piper, P. J. & Tippins, J. R. (1980) *Nature (London)* **285**, 104–106.
15. Lewis, R. A., Drazen, J. M., Austen, K. F., Clark, D. A. & Corey, E. J. (1980) *Biochem. Biophys. Res. Commun.* **96**, 271–277.
16. Sok, D.-E., Pai, J.-K., Atrache, V. & Sih, C. J. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 6481–6485.
17. Houglum, J., Pai, J.-K., Atrache, V., Sok, D.-E. & Sih, C. J. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 5688–5692.
18. Miles, A. A. & Miles, E. M. (1952) *J. Physiol. (London)* **118**, 228–257.
19. Fulton, G. P., Jackson, R. G. & Lutz, B. R. (1947) *Science* **105**, 361–362.
20. Duling, B. R. (1973) *Microvascular Research* **5**, 423–429.
21. Svensjö, E., Arfors, K.-E., Arturson, G. & Rutili, G. (1978) *Upsala J. Med. Sci.* **83**, 71–79.
22. Del Maestro, F. F., Thaw, H. H., Björk, J., Planker, M. & Arfors, K.-E. (1980) *Acta Physiol. Scand. Suppl.* **492**, 43–57.
23. Arfors, K.-E., Bergqvist, D., Intaglietta, M. & Westergren, B. (1975) *Upsala J. Med. Sci.* **80**, 27–33.
24. Borgeat, P. & Samuelsson, B. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 2148–2152.
25. Örning, L. & Hammarström, S. (1980) *J. Biol. Chem.* **255**, 8023–8026.
26. Atherton, A. & Born, G. V. R. (1972) *J. Physiol. (London)* **222**, 447–474.
27. Majno, G., Palade, G. E. & Schoefl, G. I. (1961) *J. Biophys. Biochem. Cytol.* **11**, 607–626.
28. Dahlén, S.-E., Hedqvist, P., Hammarström, S. & Samuelsson, B. (1980) *Nature (London)* **288**, 484–486.
29. Grant, L. (1973) in *The Inflammatory Process*, eds. Zweifach, B. W., Grant, L. & McCluskey, R. T. (Academic, New York), Vol. 2, pp. 205–249.
30. Rouzer, C. A., Scott, W. A., Cohn, Z. A., Blackburn, P. & Manning, J. M. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 4928–4932.
31. Zweifach, B. W. (1973) in *The Inflammation Process*, eds. Zweifach, B. W., Grant, L. & McCluskey, R. T. (Academic, New York), Vol. 2, pp. 3–46.
32. Ford-Hutchinson, A. W., Bray, M. A., Doig, M. V., Shipley, M. E. & Smith, M. J. H. (1980) *Nature (London)* **286**, 264–265.
33. Smith, M. J. H., Ford-Hutchinson, A. W. & Bray, M. A. (1980) *J. Pharm. Pharmacol.* **32**, 517–518.
34. Palmer, R. M. J., Stephney, R. J., Higgs, G. A. & Eakins, K.-E. (1980) *Prostaglandins* **20**, 411–418.
35. Malmsten, C. L., Palmblad, J., Udén, A.-M., Rådmark, O., Engstedt, L. & Samuelsson, B. (1980) *Acta Physiol. Scand.* **110**, 449–451.
36. Hoover, R. L., Briggs, R. T. & Karnovsky, M. J. (1978) *Cell* **14**, 423–428.
37. Clark, E. R. & Clark, E. L. (1935) *Am. J. Anat.* **57**, 385–438.
38. Majno, G., Shea, S. M. & Leventhal, M. (1969) *J. Cell. Biol.* **42**, 647–672.
39. Holme, G., Brunet, G., Piechuta, H., Masson, P., Girard, Y. & Rokach, J. (1980) *Prostaglandins* **20**, 717–728.