Biochemical Pharmacology, Vol. 34, No. 19, pp. 3495–3498, 1985
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00
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DISAPPEARANCE AND METABOLISM OF LEUKOTRIENE B_4 DURING CARRAGEENAN-INDUCED PLEURISY

BRUCE M. TAYLOR* and FRANK F. SUN
Lipids Research, The Upjohn Co., Kalamazoo, MI 49001, U.S.A.

(*Received* 14 *September* 1984; *accepted* 27 *February* 1985)

Abstract—Leukotriene B_4 (LTB_4) has been implicated as a mediator in the inflammatory process by virtue of its potent chemotactic activity. At present, very little is known of the stability of this compound *in vivo*; therefore, the present study was designed to determine the half-life and metabolic fate of radiolabeled LTB_4 during a 2-hr intrapleural incubation in rats with acute carrageenan pleurisy. After injection of 0.2 ml of 1% sodium carrageenan (Viscarin), inflammation was allowed to develop for 4 hr. A small polyethylene cannula was then inserted into the chest, and 0.1 μCi of [^{14}C]LTB_4 was injected into the chest. Samples for radioactivity determination were taken at 0, 1, 2, 3, 4, 5, 7, 10, 15, 20, 30, 45, 60, 90 and 120 min via the cannula, and at 120 min the entire content of the chest was collected. The half-life for the disappearance of radioactivity from the chest was 45.8 ± 3.5 min. The 120-min samples were treated with acetone to precipitate protein and extracted with Sep-Paks. The extracts were analyzed by reversed phase high performance liquid chromatography using an ultraviolet detector set at 269 nm and a radioactivity detector. An additional experiment was run using multi-[^{3}H]LTB_4, and the only major metabolites detected were ω-hydroxylated compounds. It can be concluded from these results that LTB_4 is relatively stable *in vivo* and could be present for long enough at the inflammatory site to have an influence upon inflammatory cell migration.

Leukotriene B_4 (LTB_4) is a lipoxygenase product produced by polymorphonuclear leukocytes which is a potent chemotactic factor for leukocytes both *in vitro* [1] and *in vivo* [2] when administered exogenously. The presence of this compound has been reported in several pathologic states. Cromwell *et al.* [3] detected LTB_4 in the sputum of patients with cystic fibrosis by high performance liquid chromatography (HPLC) and *in vitro* neutrophil migration assay. LTB_4 was implied to be the chemotactic factor responsible for the increased Boyden chamber chemotaxis observed when aqueous extracts of the dermal scales from psoriasis patients were assayed, although further characterization was not attempted in this study [4]. A third condition in which LTB_4 has been detected via the use of HPLC and neutrophil migration assay is gout by Rae *et al.* [5]. Klickstein *et al.* [6] assayed synovial fluid from arthritic knees by HPLC analysis using u.v. detection and found LTB_4 to be present. However, in a more recent study, Davidson *et al.* [7] examined synovial fluid from arthritic patients and could find only traces of LTB_4 by both HPLC and neutrophil aggregation bioassay. From this study it was concluded that because only traces of LTB_4 could be found in the joint fluid this compound must be rapidly metabolized by the inflammatory cells present in the exudate. In order for LTB_4 to function as a major chemotactic factor, it must be sufficiently stable *in vivo* at the site of inflammation for a long enough period to produce a concentration gradient to induce leukocyte movement into the site. Therefore, the purpose of this present study was to determine the disappearance and biological stability of radiolabeled LTB_4 at a site of acute inflammation.

* Author to whom all correspondence should be addressed.

METHODS AND MATERIALS

[1-^{14}C]LTB_4 was produced by incubation of [1-^{14}C]arachidonic acid (59 mCi/mmole) (New England Nuclear, Boston, MA) with human neutrophils in the presence of the calcium ionophore A23187 (Sigma Chemical, St. Louis, MO) by the method of Sun and McGuire [8].

Determination of [^{14}C]LTB_4 *disappearance rate.* Carrageenan pleurisy was induced in 200–250 g female rats by the injection of 0.5 ml of 1% sodium carrageenan (Viscarin 402, Marine Colloids Div., FMC Co., Springfield, NJ, prepared in 0.9% saline) through a small incision in the chest wall. The incision was then closed, the rat rotated to ensure mixing of the chest contents, and inflammation allowed to develop for 20 min, 2 hr or 4 hr. Twenty minutes before the end of this development period, the animals were anesthetized with metofane (Pitman Moore, Inc., Washington Crossing, NJ) and maintained in this state for the duration of the experiment. Following anesthetization, a PE-20 polyethylene cannula (10 cm long, adapted to accept a 22-g needle at one end [9] and a sleeve of shrinkable teflon tubing (3 mm long) placed 4 mm from the other end to serve as an anchor; a small notch was cut near this end to help assure patency (see Fig. 1)) was inserted through the diaphragm from the abdominal side with the aid of a wire trocar inserted through its lumen. Eastman 910 cement (Eastman Kodak, Rochester, NY) was used to secure the cannula at the teflon tubing to the abdominal wall. This cannula allowed for drug

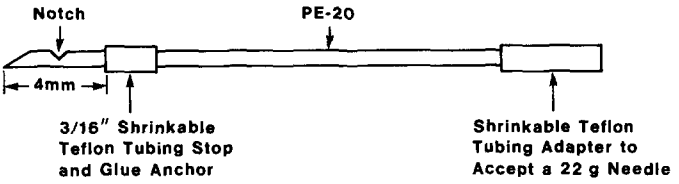

Fig. 1. Cannula used to sample chest contents.

administration into the chest and sampling of the thoracic contents.

Following these various periods of inflammatory development, 0.1 μCi (5.41 ng) of [1-^{14}C]LTB$_4$ dissolved in 0.5 ml of phosphate-buffered saline (PBS) was administered via the cannula and washed in with 50 μl of PBS, the rat was rotated to mix the chest contents, and a 25-μl sample of the chest contents was taken for radioactivity determination. Subsequent 25-μl samples were withdrawn in order to follow radioactivity disappearance at 1, 2, 3, 4, 5, 7, 10, 15, 20, 30, 45, 60, 90 and 120 min. Just prior to withdrawal of each sample, the 20-μl dead volume of the cannula was withdrawn and reinjected following each sampling. The chest samples were transferred to scintillation vials containing 15 ml ACS (Amersham, Arlington Heights, IL) and counted in a Packard 3375 scintillation spectrophotometer (Packard Instruments, Downers Grove, IL).

Metabolism of LTB$_4$ during pleurisy. After 120 min of incubation at the site of carrageenan pleurisy the animal was killed and the cells and exudate were recovered from the chest via a plastic transfer pipette. The chest cavity was washed with 2 ml PBS and the wash combined with the exudate. Proteins in the fluid samples were precipitated with 2 vol. of acetone and removed by centrifugation. Acetone in the supernatant fraction was evaporated off under reduced pressure, and the aqueous sample was acidified to pH 3 and extracted with reversed phase extraction columns (Sep-Pak C18, Waters Associates, Milford, MA). The Sep-Paks were washed with water and hexane prior to elution of the radioactivity with ethyl acetate. The radioactive components in the extract were analyzed by HPLC with a Varian 5000 HPLC (Varian Instruments, Walnut Creek, CA) fitted with an Altex Ultrasphere ODS column (Beckman Instruments, Berkley, CA). A gradient program was used for the elution of LTB$_4$ metabolites consisting of three solvents: (A) 0.08% acetic acid, (B) 50:50:0.08 methanol–water–acetic acid, and (C) methanol. The gradient was run as follows: a 30-min linear gradient was run from 25A:75B (75% water) to 24B:76C (88% methanol); this composition was then held for 10 min followed by a 5-min gradient to 100% C. Flow was maintained at 1.0 ml/min. The eluent was monitored by two detectors; a Varian Vari-chrom UV detector set at 269 nm and a Flo-One/HS radioactivity flow detector (Radiomatic Instruments & Chemical Co., Tampa, FL). The Flo-One was equipped with a 2.5-ml flow cell, and 3 ml/min Flo-Scint II scintillation fluid (Radiomatic) was mixed with the column effluent prior to entry into the flow cell. The retention times of the radioactivity peaks were compared to those of authentic standards.

Determination of time course of cell migration. To determine the time course of cell migration into the chest in response to carrageenan administration, groups of six 200–250 g female rats were injected intrapleurally with 0.2 ml of 1% sodium carrageenan. Inflammation was allowed to develop for 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5 and 6 hr in the different groups of rats. At the end of the incubation periods, rats were killed by exsanguination, their chests were opened and the entire pleural contents were washed out with heparinized PBS (10 I.U./ml) containing 1 mM EDTA. Contaminating red cells were removed by hypotonic lysis, and the infiltrated neutrophils were counted on a Coulter counter (model ZBI, Coulter Electronics, Hialeah, FL).

RESULTS

The results of the cell migration experiment are presented in Fig. 2. As can be seen from this curve, there was a 1.5 hr lag before an appreciable increase in cell number was observed over the resting level of $7.8 \times 10^6 \pm 0.66 \times 10^6$ cells normally present in the pleural cavity. A rapid influx of cells occurred from this point (1.5 hr) through 4 hr post-carrageenan administration at which point $109.7 \times 10^6 \pm 10.16 \times 10^6$ cells were present. After 4 hr, the influx of neutrophils leveled off. It should be noted here that the addition of exogenous LTB$_4$ to the chest cavity in these experiments caused no significant change in the time course of migration or the total number of cells in the chest, an observation which is not surprising since the carrageenan is probably caused a maximal chemotactic response into the thoracic cavity.

LTB$_4$ radioactivity disappearance from the chest was monitored for 2 hr following [1-^{14}C]LTB$_4$ administration from animals during three stages of carrageenan pleurisy development—20 min, a point before which cell numbers began to increase; 2 hr, when cell influx is beginning; and 4 hr, at the end of most cell migration—in order to see if the phase of cell migration had any effect on LTB$_4$ disappearance curves at the different administration times (20, 120, or 240 min). The curves from the three different administration times were superimposable; therefore, the data from all these experiments was pooled and is presented in Fig. 3. There are two phases with different disappearance rates seen in this figure: an early rapid phase with a half-life of 17.8 min and an extended phase with a half-life of 77 min. At the end of the 2-hr LTB$_4$ incubation, $17.0 \pm 2.1\%$ of the initial radioactivity was still present in the chest.

After the 2-hr incubation, the chest contents were washed out and extracted with Sep-Paks. The recovery from these extractions was $73.1 \pm 1.9\%$. An

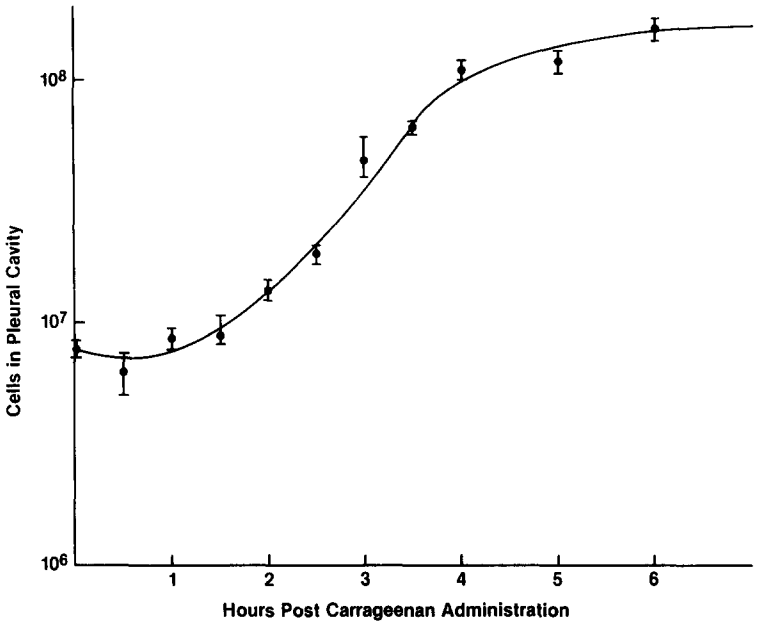

Fig. 2. Time course of cell migration into the chest during carrageenan-induced pleurisy.

HPLC radiochromatogram of this extract is presented in Fig. 4. After 2 hr of incubation in the chest, 85% of the radioactivity still cochromatographed with LTB_4. The only major metabolite detected was 20-OH-LTB_4 which accounted for 10.5% of the radioactivity, with a small amount of Δ^6-*trans*-LTB_4 also being present.

These results were further confirmed by following the metabolite profile present following administration of [14,15-^{3}H]leukotriene B_4 (New England Nuclear) to the chest during pleurisy. The metabolite pattern, as observed via HPLC analysis, was identical to that obtained from the [^{14}C]LTB_4 experiments.

DISCUSSION

Carrageenan pleurisy is a well-studied and widely-used acute inflammation model for the evaluation of potential anti-inflammatory drugs. However, the involvement of LTB_4 as a mediator of cellular infiltration has not been rigorously confirmed. Simmons *et al.* [10] used radioimmunoassay and found a small peak of LTB_4 in the carrageenan-induced inflammatory exudate just before the cell infiltration started. Our attempts to confirm this observation with HPLC or mass spectrometric methods have not been successful due to the lack of assay sensitivity. We suspected that the LTB_4 produced might be rapidly removed by metabolic processes, but from the data presented here it can be concluded that LTB_4 has a relatively long half-life at an inflammatory site, since most of the radioactivity present in the chest 2 hr post-LTB_4 administration was still associated with intact LTB_4. Metabolism of this leu-

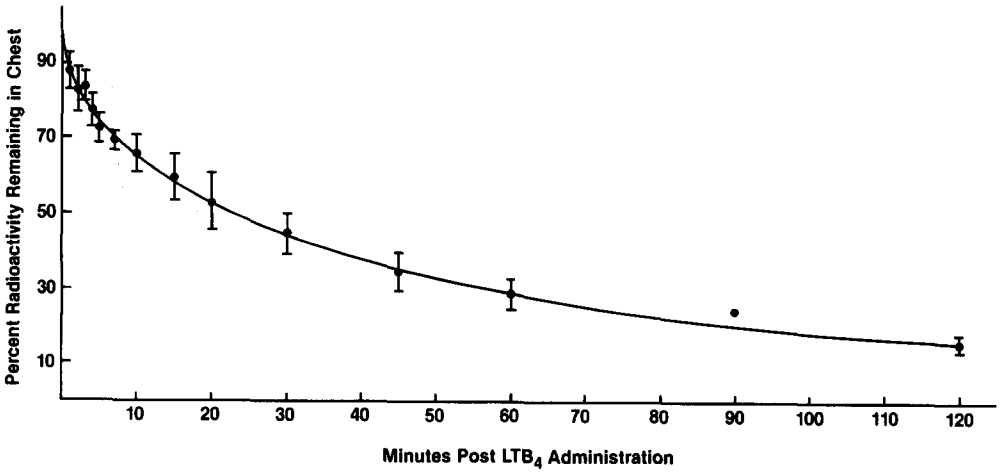

Fig. 3. Disappearance of radioactivity from the chest following administration of labeled LTB_4 to rats undergoing carrageenan-induced pleurisy.

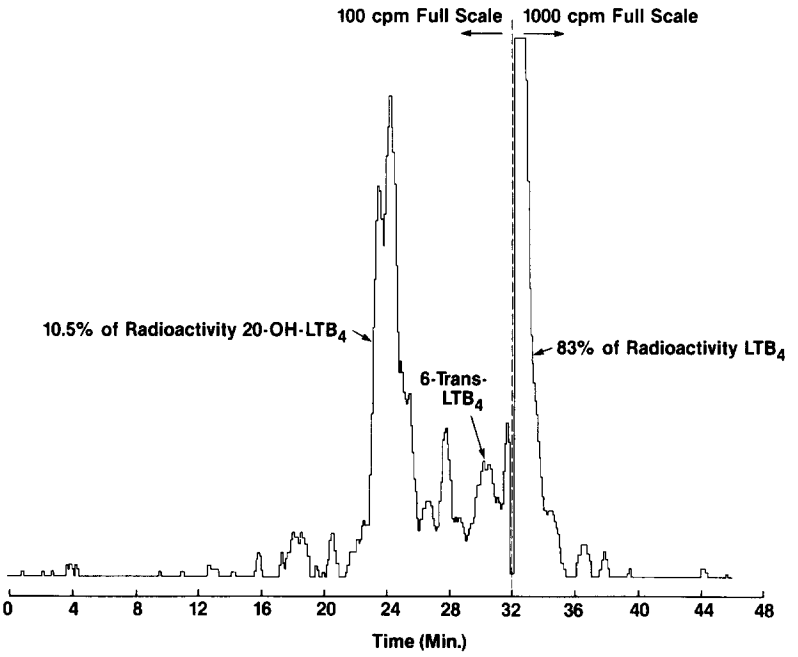

Fig. 4. Radiochromatogram showing the composition of radiolabeled LTB_4 after 120 min in the chest of rats undergoing carrageenan-induced pleurisy.

kotriene in the chest appears not to be a major route of inactivation since only two metabolites were detected, 20-OH-LTB_4 and Δ^6-*trans*-LTB_4, which accounted for only 10.5% of the total radioactivity present in the chest after 2 hr.

These data are further supported by findings of Serafin *et al.* [11] who studied whole-body metabolism of LTB_4 in both the monkey and the rabbit. According to their study, β-oxidation is the major route of inactivation of LTB_4 since most (>70%) of the radioactivity recovered in the urine following administration of [^{3}H]LTB_4 was associated with tritiated water. Indeed, the only significant metabolite identified was 20-OH-LTB_4 which is exactly what we found in the pleural cavity.

The stability of LTB_4 during pleurisy may be due to protein binding *in vivo* which has been reported to stabilize other labile prostanoids *in vivo* [12]. In addition, Fitzpatrick *et al.* [13] have studied the effect of albumin on the stability of LTA_4, the unstable intermediate in the lipoxygenase pathway, *in vitro*. Their findings indicate that the stability of LTA_4 increases as a function of albumin concentration and that the stabilizing concentration of the protein is well below its normal physiological concentration. This finding would also be expected to hold true for LTB_4.

In conclusion, we found that if LTB_4 is produced at the site of carrageenan inflammation it should be present for sufficiently long duration to affect leukocyte chemotaxis.

REFERENCES

1. A. W. Ford-Hutchinson, M. A. Bray, M. W. Doig, M. E. Shipley and M. J. H. Smith, *Nature, Lond.* **286**, 264 (1980).
2. M. A. Bray, A. W. Ford-Hutchinson and M. J. H. Smith, *Prostaglandins* **22**, 213 (1981).
3. O. Cromwell, H. R. Morris, M. E. Hodson, M. J. Walport, G. W. Taylor, J. Batten and A. B. Kay, *Lancet* **25**, 164 (1981).
4. J. Grabbe, B. M. Czarnetzki and M. Mardin, *Lancet* **25**, 1464 (1982).
5. S. A. Rae, E. M. Davidson and M. J. H. Smith, *Lancet* **20**, 1122 (1982).
6. L. B. Klickstein, C. Shapleigh and E. J. Goetzl, *J. clin. Invest.* **66**, 1166 (1980).
7. E. M. Davidson, S. A. Rae and M. J. H. Smith, *J. Pharm. Pharmac.* **34**, 410 (1982).
8. F. F. Sun and J. C. McGuire, *Prostaglandins* **26**, 211 (1983).
9. J. R. Weeks, in *Methods in Psychology* (Ed. R. O. Myers), Vol. 2, pp. 155–68. Academic Press, London (1972).
10. P. M. Simmons, J. A. Salmon and S. Murcada, *Biochem. Pharmac.* **32**, 1353 (1983).
11. W. E. Serafin, J. A. Oates and W. C. Hubbard, *Prostaglandins* **27**, 899 (1984).
12. M. A. Wynalda and F. A. Fitzpatrick, *Prostaglandins* **20**, 853 (1980).
13. F. A. Fitzpatrick, W. F. Liggett, D. R. Morton and J. E. Pike, in *Leukotrienes and Other Lipoxygenase Products* (Ed. P. J. Piper), p. 78. Research Studies Press (1983).