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THE RELEASE OF LEUKOTRIENE B_4 DURING EXPERIMENTAL INFLAMMATION

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Abstract—Leukotriene B_4 (LTB_4) has been detected by radioimmunoassay in inflammatory exudates obtained following the implantation of saline- or carrageenan-soaked polyester sponges in rats. The immunoreactive material was confirmed as LTB_4 after extraction and purification by high pressure liquid chromatography. The peak concentration (6.9 ± 0.5 ng/ml) was detected 6 hr after implantation of sponges soaked in 0.5% carrageenan; thereafter the level declined and was undetectable after 16–24 hr. The concentration of LTB_4 during the early phase of the inflammatory response (4–8 hr) is sufficient to induce leukocyte aggregation, chemotaxis and degranulation of polymorphonuclear leukocytes (PMN) *in vitro*. Therefore, LTB_4 may mediate, at least in part, the influx of PMN and contribute to other events which characterise the inflammatory response. The level of thromboxane B_2 (TXB_2) in the inflammatory exudate followed a similar time-course to that of LTB_4 although the maximum concentration was higher (15–30 ng/ml). However, prostaglandin E_2 (PGE_2) exhibited a different time-course; the maximum level (20–30 ng/ml) was also reached 6–8 hr after implantation but remained elevated at 24 hr. The PMN count in the sponges and the concentrations of both LTB_4 and TXB_2, but not PGE_2, were significantly reduced by prior treatment of the animals with colchicine. This suggests that PMN are the major source of LTB_4 and TXB_2 in the inflammatory exudate whereas PGE_2 is produced in significant amounts by other tissues.

Leukotriene B_4 (LTB_4) [5*S*,12*R*-dihydroxy-6,14-*cis*-8,10-*trans* eicosatetraenoic acid] is formed enzymatically from arachidonic acid by polymorphonuclear leukocytes (PMN) [1–3]. It has been proposed that LTB_4 mediates leukocyte recruitment during inflammation since it is a potent chemokinetic and chemotactic agent for PMN of various species *in vitro* and *in vivo* [4–8]. Wedmore and Williams [9] have suggested that PMN may regulate fluid efflux from venules, thus implying that leukotactic agents, such as LTB_4, may also indirectly increase tissue oedema. Indeed, a combination of LTB_4 with a vasodilator prostaglandin, PGE_2, does significantly increase plasma leakage [7, 10]. LTB_4 may, therefore, amplify the inflammatory reaction by increasing cell infiltration which, in turn, may potentiate oedema formation. However, in order to establish LTB_4 as an inflammatory mediator it is necessary to demonstrate that it satisfies the classical criteria [11], which include that it should be 'demonstrably released or present' during the inflammatory response. Klickstein *et al.* [12], using u.v. absorption measurements after high-pressure liquid chromatography (HPLC), detected LTB_4 (141 ± 34 ng/ml) in synovial fluid from patients with rheumatoid arthritis: This concentration of LTB_4 would certainly be sufficient for biological activity and, if confirmed, would strongly suggest that LTB_4 participates as an inflammatory mediator in rheumatoid arthritis. However, other investigators [13] failed to detect LTB_4 in synovial fluid from rheumatoid patients using an identical procedure and only low levels of LTB_4 (0.34 ± 0.14 ng/ml) were measured when a more sensitive bioassay was employed. Clearly, there are methodological and/or other problems associated with LTB_4 analysis in biological samples. Recently, we developed a radioimmunoassay (RIA) for LTB_4 which is both sensitive and specific [14] and we have now utilized this assay to monitor the formation of LTB_4 in an animal model of inflammation. The model employed was the subcutaneous implantation of a sterile polyester sponge in rats; this leads to leukocyte infiltration and the presence of prostaglandin E_2 (PGE_2), thromboxane B_2 (TXB_2) and 6-keto-$PGF_{1\alpha}$ in the exudate [15]. In the present study we have measured the concentration of LTB_4 in the inflammatory exudate and compared it with production of the cyclo-oxygenase products and changes in leukocyte count.

MATERIALS AND METHODS

Materials

Heparin (Pularin), λ-carrageenan (Viscarin) and polyester sponge (thickness 0.5 cm) were purchased from Duncan, Flockhart & Co. (London, U.K.) Marine Colloids (Springfield, NJ) and Transatlantic Plastics Ltd (Surbiton, U.K.) respectively. Hank's balanced salt solution was obtained from Wellcome Diagnostics (Hither Green, U.K.). Silicic acid (60–200 mesh), oyster glycogen (type II), and HEPES buffer were all purchased from Sigma Chemical Co. (St. Louis, MO). Analytical grade and HPLC grade solvents were obtained from BDH (Poole, U.K.) and Rathburn Chemicals Ltd (Walkerburn, U.K.) respectively. [^{3}H]LTB_4 (30 Ci/mmole) was a gift from Dr D. Copsey (Amersham International, Amersham, U.K.); the purity of this tracer

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was greater than 90% as judged by reverse-phase and straight-phase HPLC. A stock solution (2.5 mg/ml in ethanol) of divalent cation ionophore A23187 (Calbiochem, Bishop's Stortford, U.K.) was stored at 4°; the stock solution was diluted before use in HEPES-buffered Hank's balanced salt solution (pH 7.4) (HHBS).

Inflammatory exudates

Sterile polyester sponges (3.5 × 1 × 0.5 cm) soaked in either 0.9% (w/v) sterile saline or 0.5% carrageenan (w/v sterile saline) were implanted subcutaneously in male Wistar rats (200–250 g) as previously described [16]. Animals were killed at various times (1–144 hr) after sponge implantation and the sponges dissected out; the inflammatory exudate was carefully squeezed from the sponge into a polypropylene tube. An aliquot (40 μl) of the exudate was removed immediately for determination of the leukocyte count using a model ZBI Coulter counter (Coulter Associates); in some cases a differential cell count was also performed. The remainder of the exudate was centrifuged at 12,000 *g* for 30 sec in a micro-centrifuge [type 320 (Buckard Scientific)] to precipitate cells and debris. The cells were removed immediately to eliminate metabolism of pre-formed LTB_4 by the PMN [17].

Assay of eicosanoids

RIA. LTB_4 in the exudate supernatant (diluted 1:2–1:5) was measured by a specific RIA [14] without prior extraction or chromatography. Preliminary experiments, in which known amounts of LTB_4 (0.2–50 ng/ml) were added to inflammatory exudate, established that the concentration of LTB_4 in exudate could be accurately determined by direct RIA. In order to confirm the identity of immunoreactive LTB_4, some samples were extracted and purified as described later, prior to RIA. The concentrations of PGE_2 and TXB_2 in the exudate supernatant, diluted in assay buffer (1:10–1:100), were also determined by RIA; the specificity of the antisera and details of the procedure were reported previously [15, 18].

Extraction and chromatography. Cell-free exudates obtained from 10 animals 6 hr after implantation of saline-soaked sponges were pooled, as were those from a similar group 6 hr after implantation of 0.5% carrageenan-soaked sponges. Approximately 10,000 cpm [^{3}H]LTB_4 was added to each sample to enable estimation of extraction efficiency. LTB_4 in the samples was extracted as previously described [14]; briefly, this involved protein precipitation with acetone, removal of neutral lipids in hexane after adjusting the aqueous acetone layer to pH 11, followed by chloroform extraction of acidic lipids, including LTB_4, from the remaining aqueous acetone after adjustment to pH 3.

The chloroform extract was taken to dryness under reduced pressure. The residue was dissolved in methanol (1 ml) and then silicic acid (200 mg) was added. After removal of the methanol under reduced pressure, the silicic acid was transferred to a glass column containing a further 800 mg silicic acid. The column was eluted with 15% ether in hexane (fraction 1) followed by 5% methanol in ethyl acetate (fraction 2).

After evaporation of solvent, fraction 2 was subjected to reverse-phase (RP) HPLC on a Zorbax C-8 column (Dupont Instruments) using a mobile phase of methanol–water–acetic acid [70:30:0.01 (v/v/v)] as previously described [14]. A variable wavelength detector [Spectromonitor III (Laboratory Data Control)] was used to monitor the absorption of the eluate at 271 nm. Eluate was collected at 20-sec intervals; the radioactivity in an aliquot (50 μl) was measured by conventional liquid scintillation techniques and immunoreactive LTB_4 in a further aliquot (10 μl) was determined by RIA. Also, leukocyte aggregating activity in pooled 2-min fractions was assessed (see later).

Leukocyte aggregation

The pro-aggregatory activity of the HPLC fractions was estimated by a modification of the method described by Cunningham *et al.* [19]. Oyster glycogen (20 ml of a 0.2% solution in saline) was administered to rats by intraperitoneal injection. After 18 hr, the leukocytes were harvested by peritoneal lavage with 20 ml HHBS containing heparin (20 I.U./ml). Cells from 10 rats were pooled and centrifuged at 100 *g* for 10 min. The cell pellet was resuspended in heparin-free HHBS followed by a repeat centrifugation. These washed cells were resuspended at a concentration of 1×10^7 cells/ml and stored at 20° until required.

Cell suspensions were warmed to 37° and stirred at 800 rpm in a dual channel optical aggregometer (Payton Associates) for 2.5 min before addition of standard LTB_4 or sample in dimethyl sulphoxide (DMSO)–HHBS [final DMSO concentration did not exceed 0.1% (v/v)]. Aggregating activity in the samples was measured as the maximum increase in light transmittance occurring after 3 min and was compared to the response of authentic LTB_4.

Stimulation of inflammatory cells with A23187

After at least 45 min pre-incubation of cells at 37°, A23187 (5 μg in 10 μl HHBS) was added to exudate samples (500 μl). The samples were mixed gently and incubation continued for 5 min at 37°. The reaction was terminated by centrifugation at 12,000 *g* for 30 sec; the supernatant was removed, diluted 1:5–1:20 in assay buffer and the concentration of LTB_4 determined by RIA.

Effect of colchicine

A group of rats (N = 5) was treated with colchicine (1 mg/kg) given by subcutaneous injection 15 min before implantation of sponges soaked in 0.5 % carrageenan. After 6 hr, the sponges were removed, and then the leukocyte count and the concentration of eicosanoids in the exudate samples were determined as described earlier. A matched control group was processed at the same time.

RESULTS

Cell infiltration

Both saline- and 0.5% carrageenan-soaked polyester sponges induced an influx of leukocytes beginning

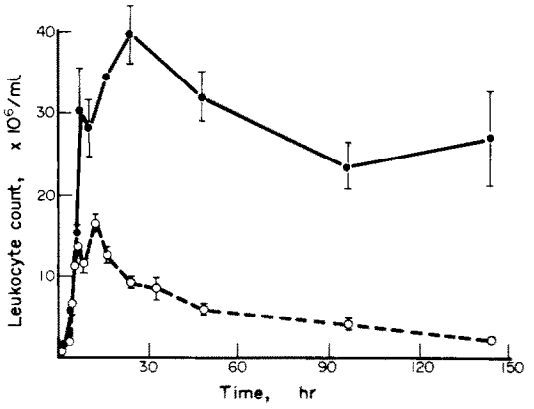

Fig. 1. Infiltration of PMN into inflammatory exudate induced by implanting polyester sponges soaked in 0.9% saline (--○--) or 0.5% carrageenan (—●—) subcutaneously in rats for various times (1–144 hr). Each point is the mean ± S.E. of 5–50 determinations.

2–3 hr after implantation (Fig. 1). However, the peak cell count (mean 16×10^6 cells/ml) was achieved at 12 hr with saline-soaked sponges whereas the cell number continued to increase in animals with carrageenan-soaked sponges and did not reach a maximum (mean 40×10^6 cells/ml) until 24 hr. The cell number remained elevated for at least 6 days after implantation of carrageenan sponges, at which time the cell count in animals given saline-soaked sponges had fallen to approximately 10% of the maximum achieved with these sponges. The predominant cell type in all samples (1–144 hr) from both groups of animals was PMN (> 95% of total leukocyte count).

Concentration of LTB_4 in inflammatory exudate

Initial experiments with exudates obtained by implantation of sponges soaked in 2% carrageenan gave inconsistent data; frequently the exudate contained a high proportion of damaged cells which correlated with a high concentration of immunoreactive LTB_4. However, there was little variation in the concentration of LTB_4 in unextracted exudate obtained from sponges soaked in either saline or 0.5% carrageenan (Fig. 2). Maximum levels of LTB_4 were achieved 4–8 hr after sponge implantation; thereafter, there was a marked decline in concentration: no LTB_4 was detected (< 0.3 ng/ml) after 16 hr. The peak concentration of LTB_4 in exudate from carrageenan sponges was higher (6.90 ± 0.52 ng/ml, N = 45) than that from saline-soaked sponges (1.67 ± 0.43 ng/ml, N = 5). The different concentration of LTB_4 in the two sponge types was also noted when expressed as ng $LTB_4/10^6$ cells; the mean maximum level of LTB_4 in saline and 0.5% carrageenan-soaked sponge exudate was 0.12 ± 0.02 (N = 5) and 0.50 ± 0.04 (N = 45) ng/10^6 cells respectively ($P < 0.01$).

The immunoreactivity in exudate supernatant

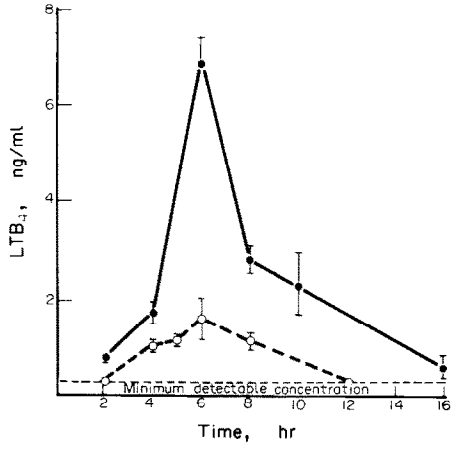

Fig. 2. Concentration of immunoreactive LTB_4 in inflammatory exudate obtained after implanting 0.9% saline (--○--) or 0.5% carrageenan- (—●—) soaked sponges in rats. Each point is the mean ± S.E. of 5–45 observations.

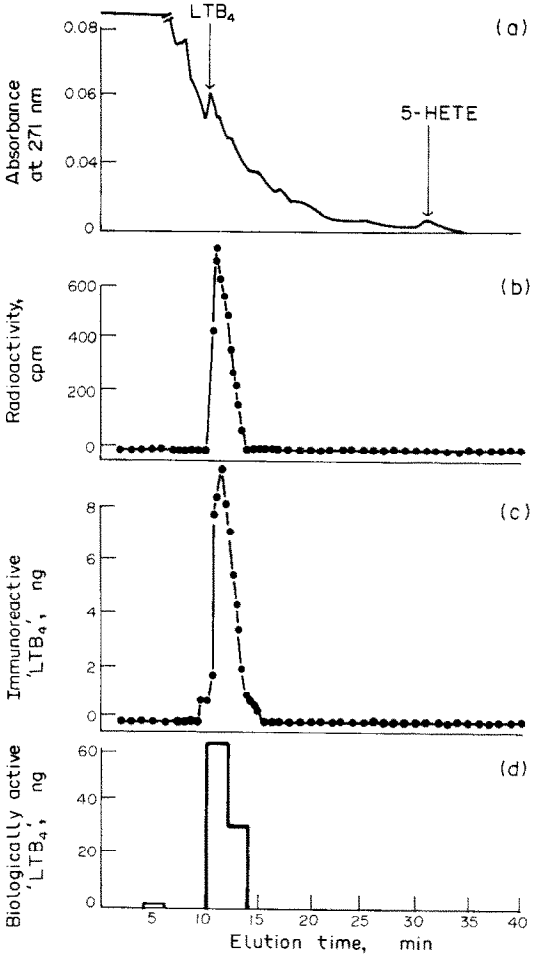

Fig. 3. Separation by HPLC of LTB_4 extracted from pooled inflammatory exudate obtained from 10 rats 6 hr after implantation of 0.5% carrageenan-soaked sponges. Absorbance of the column eluate at 271 nm is shown in panel a. Fractions of eluate (20-sec) were collected to permit estimation of: (b) [^{3}H]LTB_4 added to monitor extraction and chromatography, and (c) immunoreactive LTB_4. Also, leukocyte-aggregating activity in pooled 2-min fractions is shown in panel d.

from both sponge types was confirmed to be LTB_4 following extraction, silicic acid chromatography and HPLC (Fig. 3). The major HPLC peak of immunoreactivity extracted from the exudate occurred at a retention volume identical to that of authentic LTB_4; it was also associated with the elution of: (i) a small peak of absorbance at 271 nm, (ii) [^{3}H]LTB_4, and (iii) leukocyte-aggregating activity (Fig. 3). The overall recovery of [^{3}H]LTB_4 was 40–55%. A small amount of immunoreactive material eluted immediately preceding the peak of LTB_4 and this suggests the presence of a low concentration of 6-*trans*-LTB_4 [14]. Immunoreactive LTB_4 was not detected in either similarly processed cell pellets derived from inflammatory exudates or in buffer blanks. As noted previously [14], the tracer added to monitor the efficiency of the aforementioned extraction and chromatographic procedures may limit the accuracy of quantitative data obtained by subsequent RIA; however, the additional radioactivity in the aliquots of HPLC fractions analysed by RIA was less than 50 cpm compared with 3000–3500 cpm added in the RIA itself. It should be emphasised that other quantitative data reported in this paper were obtained by direct RIA of the exudates and the experiments, in which immunoreactive LTB_4 was measured after extraction and chromatographic purification, only serve to confirm the specificity of the assay.

As mentioned earlier, fractions from the HPLC which contained immunoreactive LTB_4 also induced leukocyte aggregation (Fig. 3). The dose–response curve for the activity in these fractions was parallel to that of authentic LTB_4, strongly suggesting that the eluted material was indeed LTB_4. The amount of LTB_4 detected in the HPLC fractions by this bioassay (total *ca.* 90 ng) compares favourably to that measured by RIA (total *ca.* 65 ng) (Fig. 3); it should be noted that because of the lower sensitivity of the bioassay a larger fraction (2 ml) was assayed.

Concentration of PGE_2 and TXB_2 in inflammatory exudate

The concentration of PGE_2 and TXB_2 in the exudate from both types of sponge-implant reached a maximum at approximately the same time as LTB_4, although the concentration of each cyclo-oxygenase product was approximately ten times the level of LTB_4 (Fig. 4). After 4–8 hr, the concentration of TXB_2 decreased in parallel to that of LTB_4. However, the concentration of PGE_2 remained higher for 24 hr, especially in exudate derived from 0.5% carrageenan-sponge implants.

Stimulation of LTB_4 synthesis in inflammatory cells by A23187

Cells derived from inflammatory exudate obtained up to 8 hr after sponge implantation had a high capacity to synthesize LTB_4 when stimulated with A23187 (10 μg/ml). However, exudate obtained after 8 hr produced less LTB_4; at 48 hr the quantitty of LTB_4 formed was less than 10% of that produced at 8 hr. This decrease was partly attributable to a small decrease in the total cell count (Fig. 1) but was still highly significant when these data were presented as amount of LTB_4 formed/10^6 cells (Fig. 5). The decreased capacity to synthesize LTB_4 from

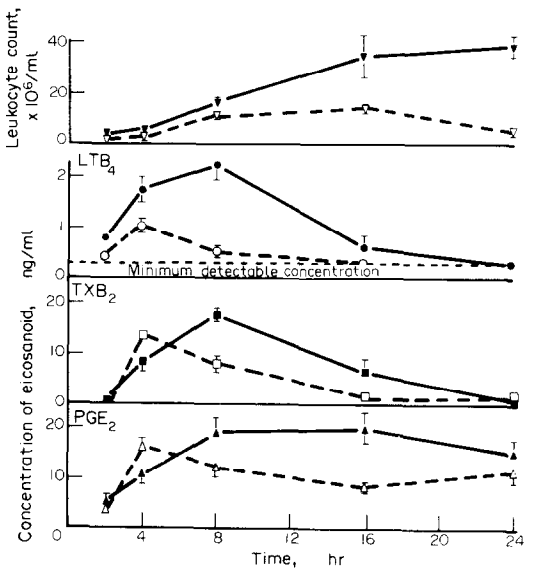

Fig. 4. Leukocyte count and concentration of eicosanoids in inflammatory exudate obtained by implanting 0.9% saline- (open symbols) or 0.5% carrageenan- (filled symbols) soaked sponges subcutaneously in rats. Each point is the mean ± S.E. of at least five observations.

endogenous arachidonic acid after A23187 stimulation occurred in exudate from both types of sponge. Addition of arachidonic acid (10 μg/ml) to exudate prior to stimulation with A23187 did not restore the capacity of the cells to synthesize LTB_4. The rate of metabolism of exogenous LTB_4 (20 ng/ml) in cells derived from 6- and 48-hr exudate was similar (half-life approximately 2 hr).

Exogenous calcium was not added to the exudate prior to treatment with A23187, and therefore the observed marked stimulation of LTB_4 synthesis by the exudate cells suggests that either the exudate contains sufficient calcium or that mobilization of intracellular rather than extracellular calcium is

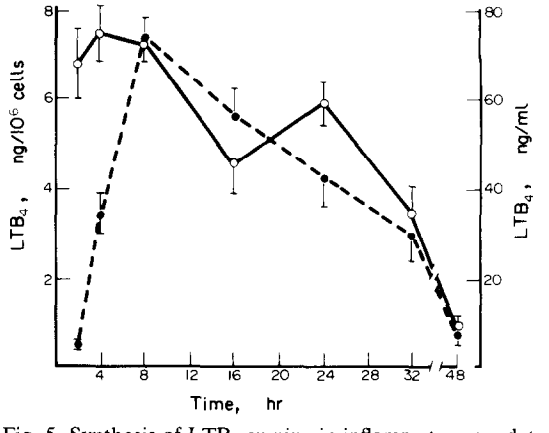

Fig. 5. Synthesis of LTB_4 *ex vivo* in inflammatory exudate (obtained by implanting 0.9% saline-soaked sponges in rats) after stimulation with A23187 (10 μg/ml). The data are expressed as ng LTB_4/10^6 cells (○——○) or ng LTB_4/ml exudate (●- - - -●). Each point is the mean ± S.E. of at least five observations.

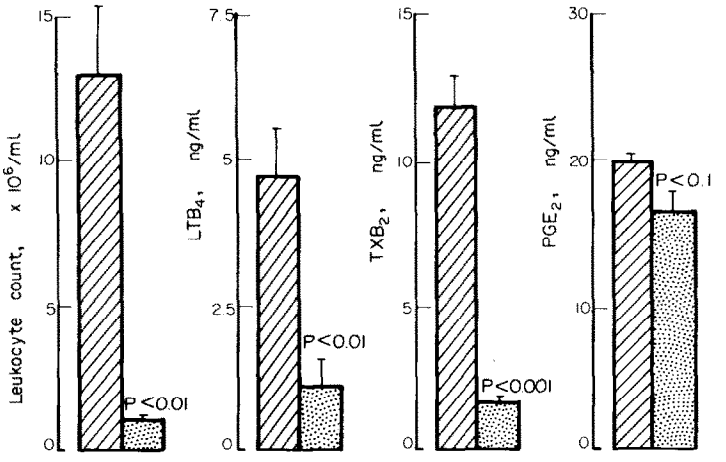

Fig. 6. Effect of colchicine (1 mg/kg s.c.) on cell infiltration and concentration of eicosanoids in inflammatory exudate obtained 6 hr after implantation of 0.5% carrageenan-soaked sponges. Control (▨) and colchicine-treated (▩) data are presented as the means ± S.E. of six and five observations respectively. The significance of the data was assessed using Student's *t*-test.

important. Addition of extra calcium (1 mM) did not further enhance LTB_4 synthesis.

Effect of colchicine

Colchicine (1 mg/kg s.c.) reduced the leukocyte count by more than 90% in exudate obtained 6 hr after implantation of 0.5% carrageenan-soaked sponges (Fig. 6). The concentration of LTB_4 and TXB_2 were similarly depressed ($P < 0.01$ and < 0.001 respectively) but the level of PGE_2 was not significantly lower than the control [$P < 0.1$ (Fig. 6)].

DISCUSSION

Using RIA, LTB_4 was detected in exudates obtained during the early stages of experimentally-induced inflammation. Quantitative determination of LTB_4 was by direct RIA of the exudate supernatant but the immunoreactive material was confirmed to be LTB_4 following extraction, silicic acid chromatography and HPLC. Thus, RIA of LTB_4 in exudate, without prior extraction or purification, offers a valid and reliable assay enabling practical analyses of large numbers of samples.

The maximum concentration of LTB_4 was detected at 6 hr, a time which approximately correlated with the *fastest rate* of leukocyte infiltration but before the time of maximum white cell count in the exudate. The concentrations of LTB_4 would be sufficient to induce chemokinesis and chemotaxis [4, 5] and degranulation [20, 21]; indeed we confirmed that material extracted from the inflammatory exudate derived from a single rat is sufficient to induce aggregation of rat leukocytes *in vitro*. Exudates obtained by implanting sponges soaked in 0.5% carrageenan contained significantly higher concentrations of LTB_4 and leukocytes than those from saline-soaked sponges. These observations suggest that LTB_4 may, at least in part, mediate cell recruitment to sites of inflammation. However, our data do not prove whether the presence of LTB_4 in the exudate is the cause or consequence of a high rate of cell infiltration. Other substances present in inflammatory exudate such as the complement-derived peptide C5a [22], will induce cell influx. It is possible that LTB_4 amplifies this chemotactic response in the early phase of inflammation.

The maximum concentration of LTB_4 in the exudate is approximately 10–30% of the concentration of both PGE_2 and TXB_2. Some stimuli such as the divalent cation ionophore A23187 [14, 23] and serum-treated zymosan [24, 25] preferentially increase LTB_4 synthesis. This suggests that the activation of arachidonic acid metabolism in PMN *in vitro* by these stimuli does not mimic the activation occuring during inflammation *in vivo*. However, LTB_4 is readily metabolized by human PMN to more polar, less biologically active compounds (via ω-oxidation to 20-hydroxy- and 20-carboxy-LTB_4 [17, 26, 27]); these metabolites do not cross-react in the RIA for LTB_4 (27). Polymorphonuclear leukocytes do not significantly metabolize the cyclo-oxygenase products, PGE_2 and TXB_2 [27]. The rate of LTB_4 metabolism by PMN may be increased in the presence of activating stimuli such as the chemotactic peptide, f-Met–Leu–Phe [28]. We have confirmed that rat PMN also metabolized LTB_4, albeit at a slower rate than human cells. Therefore, ω-oxidation of LTB_4 may explain the low concentration of unchanged LTB_4, relative to PGE_2 and TXB_2, present in the exudate and this metabolism may serve to localize and limit its duration of activity.

The concentration of both PGE_2 and TXB_2 measured in the present study follows a similar time-course to that previously described [15]. However, the concentration of both cyclo-oxygenase products reported in the latter study were higher than those presented here, probably because a higher concentration of carrageenan (2%) was employed. Sponges soaked in 2% carrageenan induce a higher total leukocyte count which is partly due to the influx of a high number of monocytes after 48 hr [29]; negligible numbers of monocytes were observed in exudates from saline or 0.5% carrageenan sponge implants. We did not study fully the exudate derived from 2% carrageenan-treated rats because we

observed an inconsistent number of damaged cells in the exudate which correlated with an increase in the concentration of LTB_4. The presence of damaged cells may reflect phagocytosis leading to autolysis *in vivo* or possibly activated cells may aggregate and rupture spontaneously producing LTB_4 *ex vivo*. Perhaps the high levels of LTB_4 reported in synovial fluid [12] may be attributable to generation of LTB_4 by the inflammatory cells *ex vivo*. Therefore, careful collection and handling of samples is mandatory for reliable measurement of the true *in vivo* concentration of LTB_4.

We observed previously [15] that the concentration of TXB_2 decreased at a time when the level of PGE_2 and the cell numbers were still increasing. The present study confirmed these data and also demonstrated that the time-course of LTB_4 production was similar to that of TXB_2, which suggests that both these eicosanoids are generated from the same cell type. On the other hand, PGE_2 is probably formed by other tissues, which maintain the high concentration of PGE_2 after the levels of both LTB_4 and TXB_2 have fallen markedly. The observation that colchicine, which inhibits leukocyte infiltration by more than 90%, significantly reduces the levels of LTB_4 and TXB_2, but not PGE_2 (present data and Ref. 30) indicates that LTB_4 and TXB_2 are biosynthesized by PMN but that other tissues are the major source of PGE_2.

On the basis of these results it is difficult to speculate about the precise function of LTB_4 in the initiation or maintenance of the cellular response in inflammation. It has been suggested that other products of arachidonic acid metabolism modulate some characteristic symptoms of inflammation (oedema, vasodilatation and hyperalgesia) by amplifying the actions of mediators such as bradykinin and 5-hydroxytryptamine (see Ref. 31). It is possible that LTB_4 acts also as a modulator of the actions of other chemotactic factors present during the inflammatory response.

The reason for the fall in LTB_4 concentration 4–8 hr after sponge implantation has not been fully investigated. We did note, however, that cells derived from sponges implanted for more than 8 hr had a reduced capacity to synthesize LTB_4 after A23187. This was not, apparently, due to decreased substrate availability because the high synthetic capacity was not restored by exogenous arachidonic acid. In preliminary experiments, we have observed that cells from 48-hr inflammatory exudates are able to synthesize more LTB_4 in response to A23187 when resuspended in buffer than if they remained in the unprocessed exudate. This suggests the presence of a factor(s) in the exudate which inhibits LTB_4 synthesis. Further work is in progress to elucidate the precise mechanism by which LTB_4 formation is 'switched off'.

In conclusion, we have established that LTB_4 is formed in acute experimentally-induced inflammation but we have not proved that it is an important mediator of leukocyte accumulation in this model. In order to clarify the role of LTB_4, we are attempting to correlate the anti-flammatory activity of drugs with their effectiveness in inhibiting LTB_4 synthesis *in vivo*.

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