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LEUKOCYTE RECRUITMENT IN THE SUBCUTANEOUS SPONGE IMPLANT MODEL OF ACUTE INFLAMMATION IN THE RAT IS NOT MEDIATED BY LEUKOTRIENE B_1

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Abstract—The subcutaneous sponge implant model of acute inflammation in the rat has been evaluated as a suitable test system for evaluating the potential anti-inflammatory efficacy of 5-lipoxygenase inhibitors. The inflammatory parameters measured were exudate volume and leukocyte recruitment. Specific radioimmunoassays were used to measure (1) 5-lipoxygenase (LPO) and cyclo-oxygenase (CO) activity in exudate leukocytes stimulated *ex vivo* with A23187, and (2) the LTB_4 and PGE_2 content of inflammatory exudate. The NSAIDs flurbiprofen and indomethacin inhibited cell recruitment, exudate volume and CO activity with ED_{50}s of approximately 1 mg per kg p.o. but failed to inhibit LPO activity at 10 mg per kg p.o. Nafazatrom (Bayer 6575), quercetin and NDGA, which inhibit LPO activity *in vitro*, were inactive against all parameters when dosed at 100 mg per kg p.o. The "mixed inhibitors" BW755C and phenidone were approximately equipotent inhibitors of LPO activity but BW755C was 10 times more potent than phenidone against CO activity. BW755C was also >10 times more potent at inhibiting cell recruitment and exudate volume than phenidone suggesting that the anti-inflammatory efficacy of the mixed inhibitors reflect their potency against CO rather than LPO activity. Time course studies demonstrated that the inhibitor effects of BW755C and phenidone on leukocyte recruitment reflected a reduction in the PGE_2 but not the LTB_4 content of the inflammatory exudate. Polyester sponges soaked in high concentrations of LTB_4 caused only a modest (2-fold) increase in leukocyte recruitment whilst physiological levels were inactive. The results taken together suggest that CO products make a major contribution to leukocyte recruitment in this model whilst the LPO product LTB_4 has little role. This model therefore is of little value for evaluating the anti-inflammatory efficacy of 5-lipoxygenase inhibitors. Moreover, the rat would appear to be unsuitable for evaluating the role of LTB_4 in acute inflammation.

Polymorphonuclear leukocytes are prominent at sites of acute inflammation. Their infiltration is stimulated in pathological conditions by a variety of agents which include bacteria, immune complexes and complement derived chemotactic peptides. In addition deposition of agents such as zymosan, kaolin, carrageenin, urate crystals and the synthetic peptide f-met-leu-Phe into tissues invoke an acute inflammatory reaction characterised by PMNL† infiltration. Recently attention was focused on the 5-lipoxygenase (EC1.13.11.12) product LTB_4 which has been demonstrated to cause the accumulation of PMNL into the guinea-pig peritoneal cavity [1]; rabbit eye [2] and skin [3–5]; sheep skin and lung [6] and human skin [7, 8]. When LTB_4 is combined with vasodilator substances such as the E-type prostaglandins or prostacyclin it invokes the leakage of plasma from the microvasculature into the tissues [9, 10], a process which has been shown to be dependent on circulating PMNL [10].

LTB_4 has been detected in inflammatory exudates taken from experimental animals [11, 12] and in patients with chronic inflammatory disease [13, 14]. The source of this LTB_4 appears to be leukocytes since these cells can be stimulated to generate the substance when stimulated with pathological stimuli such as urate crystals [15], zymosan [16] and bacteria‡ and with the non-physiological calcium ionophore A23187 [17]. Thus LTB_4 may act as a signal for amplifying the acute inflammatory response. This evidence, although circumstantial, supports a role for LTB_4 in the pathology of acute inflammation. It has been speculated therefore that agents which inhibit the biosynthesis of LTB_4 may have therapeutic benefits in the treatment of inflammatory disease. In order to evaluate the potential anti-inflammatory efficacy of such agents it is important to identify, in an animal model of inflammation, a measurable parameter such as leukocyte recruitment which can be wholly or at least partly attributable to LTB_4 generated *in situ*.

Salmon *et al.* [18] based on their investigations of the effects of several anti-inflammatory drugs on the eicosanoid and leukocyte content of the inflammatory exudate obtained from carrageenin-soaked subcutaneous sponge implants in rats have suggested that, "the reduction of cell migration by BW755C

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† Abbreviations used: PMNL, polymorphonuclear leukocytes; LTB_4, leukotriene B_4; (5*S*,12R)-5,12-dihydroxy-6-14-*cis* 8, 10-*trans*-eicosatetraenoic acid; PGE_2, prostaglandin E_2; NDGA, nordihydroguaiaretic acid; HPLC, high-pressure liquid chromatography; AA, arachidonic acid; 5-HETE, 5-hydroxy-6,8,11,14-eicosatetraenoic acid; 12-HETE, 12-hydroxy-6,8,11,14-eicosatetraenoic acid; DMSO, dimethylsulphoxide.

‡ P. A. J. Hendricks, M. E. Vandertol and J. Verhoef, *Agents Actions*, in press.

and dexamethasone could be ascribed, at least in part, to inhibition of LTB_4 synthesis". We have therefore investigated the subcutaneous sponge implant model of acute inflammation in the rat as a suitable test system for evaluating the anti-inflammatory efficacy of inhibitors of LTB_4 synthesis.

Specific RIAs for LTB_4 and PGE_2 were used to determine the eicosanoid content of the inflammatory exudate at various times following sponge implantation. The effect of inhibitors of eicosanoid biosynthesis on the leukocyte and eicosanoid content of the inflammatory exudate were assessed and the results suggest that cyclo-oxygenase (EC1.14.99.1) products make a major contribution to leukocyte recruitment in this model whilst the lipoxygenase product LTB_4 has little role. This latter point was supported by the observation that the *in vivo* chemotactic response of rats to LTB_4 was poor which implies that the rat is not a suitable species for investigating the role of LTB_4 in acute inflammation.

MATERIALS AND METHODS

Materials

Polyester sponge type E41 (thickness 0.5 cm) was purchased from Kay Metzler, Polyurethane Division, Middlesex, U.K.

Type IV carrageenin, streptomycin sulphate, calcium ionophore A23187, EGTA, Sodium azide, indomethacin, phenidone (1-phenyl-3-pyrazolidone), quercetin, dexamethasone, NDGA and PGE_2 were obtained from the Sigma London Chemical Company (Poole, U.K.). Benzylpenicillin was purchased from Glaxo (Greenford, U.K.); heparin sodium salt from Evans Medical (Speke, U.K.); sodium brietal from May and Baker (Dagenham, U.K.); carboxymethylcellulose from BDH Chemicals (Poole, U.K.) and flurbiprofen was a gift from The Boots Company PLC (Nottingham, U.K.). All solvents used were of analytical or HPLC grade. Reversed-phase HPLC columns, 25 × 0.5 cm containing 5 μ Spherisorb ODS2 were purchased from Hichrom (Reading, U.K.). The radiochemicals [^{3}H] LTB_4 (222Ci/mmol), [^{3}H] PGE_2 (160Ci/mmol), [^{14}C] arachidonic acid (58 Ci/mmol) and ^{125}I-human serum albumin (^{125}I-HSA; 100 μCi) were purchased from Amersham International (U.K.) and [^{3}H] 12-HETE (40 Ci/mmol) from NEN (Boston, MA). [^{14}C] 5-HETE was made biosynthetically using an RBL-1 5-lipoxygenase preparation by D Masters ICI Pharmaceuticals Division.

BW755C (3-amino-1-[m-trifluoromethyl)phenyl]-2-pyrazoline HCl) and nafazatrom (2,4-dihydro-5-methyl-2-[2-(naphtyloxy]ethyl-3H-pyrazol-3-one) were synthesised in the Chemistry Department at ICI Pharmaceuticals Division. Synthetic LTB_4 was prepared by Dr Y. K. Yee (Stuart Pharmaceuticals, Delaware) using a modification of the procedure of E. J. Corey *et al.* [19] as described previously [20].

Subcutaneous sponge implant procedure

Polyester sponges (3.0 × 1.0 × 0.5 cm) were boiled in ethanol for 2 hr, washed thoroughly with distilled water and steriflex (The Boots Company PLC, Nottingham, U.K.) then soaked in a 0.5% (w/v) solution of carrageenin in steriflex containing 100 μg/ml each of benzylpenicillin and streptomycin sulphate. Sponges were stored at 4° overnight prior to implantation. Male Wistar rats weighing 180–220 g were anaesthetised using sodium brietal (approx. 12.5 mg i.p.) The abdomen was swabbed with a 1% (v/v) solution of "Savlon" H.C. antiseptic (Imperial Chemical Industries plc) in 70% (v/v) ethanol/water and a 1 cm incision was made in the skin of the abdomen through which two sponges were inserted subcutaneously, one each side of the ventral line. The incision was closed using 9 mm stainless steel autoclips (Clay Adams, Parsippany, NJ).

Inflammatory exudates

At various times following sponge implantation the rats were killed in a carbon dioxide filled chamber. The sponges were removed and the exudate collected into 100 units of heparin in polypropylene centrifuge tubes (Sarstedt Ltd, Leicester, U.K.) by squeezing through a 10 ml syringe. The exudate volume was measured by weighing on a Sartorius 1219 MP balance (Sartorius GMBH, Gottingen, F.R.G.) and the total leukocyte content determined in duplicate 20 μl samples with a model ZBI Coulter Counter (Coulter Electronics, Herts, U.K.). Differential cell counts were made on smears, fixed in methanol and stained with Giemsa's stain. In some instances a portion of the exudate was centrifuged for 1 min in an Eppendorf 3200 bench centrifuge and a sample of the cell-free exudate was stored at −20° for subsequent determination of eicosanoid content by RIA.

Plasma exudation

Plasma exudation was measured by monitoring the leakage of ^{125}I-HSA into the sponge exudate. Immediately prior to sponge implantation rats were injected intravenously with 0.5 μCi of ^{125}I-HSA in 0.1 ml of saline. At various times following sponge implantation the animals were killed, the sponges were removed and a blood sample was taken into 100 units of heparin. The radio-activity of the sponges and plasma samples (200 μl) were determined using an LKB1280 Ultro-gamma counter. The volume (μl) of plasma leakage into the sponge was calculated from cpm sponge/cpm plasma sample × 200.

Stimulation of inflammatory exudate with A23187

Inflammatory exudate (250 μl) collected from 4 hr sponge implants was preincubated for 15 min at 37° prior to the addition of 5 μl of A23187 (0.5 mg/ml in DMSO). Reactions were terminated 30 min later by the addition of 50 μl of "cocktail" (1 mM BW755C, 1 mM indomethacin, 100 mM EGTA, 1% sodium azide, pH 7.2) then centrifuged for 2 min in an Eppendorf 3200 bench centrifuge. Supernatants were stored at −20° for subsequent determination of eicosanoid content by RIA.

Effect of compounds

(a) In vivo. Compounds were freshly formulated by dissolving in 1 ml of DMSO then added to 14 ml of 1% (w/v) carboxymethylcellulose and sonicated for 10 sec using an MSE ultrasonic disintegrator.

Rats (groups of 10), which were allowed access to food and water *ad libidum*, were dosed orally with test compounds one hour prior to sponge implantation. Control rats (20 per experiment) were dosed with vehicle alone. The LTB_4 and PGE_2 content of the cell free inflammatory exudate obtained, as described above, at various times following sponge implantation were determined by RIA.

(b) Ex vivo. The formation of LTB_4 and PGE_2 by A23187-stimulated leukocytes in the inflammatory exudates obtained from compound-treated rats was monitored as described above. The amount of LTB_4 and PGE_2 generated *ex vivo* was calculated as ng eicosanoid per 10^6 leukocytes. This compensates for the effect which some compounds have on the exudate leukocyte content.

(c) In vitro. Compounds, dissolved in DMSO at 100 times the test concentrations, were added to 250 μl of control exudate to give final concentrations of 10^{-9}–10^{-4} M. After 15 min preincubation at 37°, A23187 (2.5 μg in 5 μl DMSO) was added and incubation continued for 30 min. The reactions were terminated as described above and the formation of LTB_4 and PGE_2 quantitated by RIA.

Assay of eicosanoids

The LTB_4 and PGE_2 content of cell-free inflammatory exudate and A23187-stimulated exudate supernatants was measured by specific RIAs [21].

Procedure used to authenticate the nature of the immunoreactive LTB_4

Cell-free inflammatory exudate (4 ml) recovered from 0.5% carrageenin-soaked sponges 4 hr following their subcutaneous implantation into rats or the supernatant obtained after the stimulation of exudate leukocytes with A23187 for 30 min was adjusted to pH 5.6 with glacial acetic acid, centrifuged to remove insoluble material and methanol was added to give a final concentration of 40% (v/v). After centrifugation the supernatant was subjected to reversed-phase HPLC using a 5 μ Spherisorb ODS2 column and linear gradient of 40–95% methanol in 0.1% (v/v) acetic acid and water, pH 5.6. Samples (0.2 ml) were loaded at a flow rate of 0.2 ml per min for 4 min after which the rate was increased to 1.5 ml per min for elution. The reversed-phase HPLC column was calibrated using radiolabelled [^{3}H] LTB_4, [^{3}H] 12-HETE, [^{14}C]5-HETE and [^{14}C] arachidonic acid.

RESULTS

The infiltration of leukocytes into 0.5% carrageenin-soaked polyester sponges implanted subcutaneously into rats is shown in Fig. 1. After a 2 hr lag period there occurred a rapid infiltration of leukocytes which peaked (mean $30.0 \pm 2.5 \times 10^6$ cell/ml) at approximately 8–10 hr post-implant. The leukocytes then began to decline prior to a second wave of leukocyte infiltration later than 24 hr. Approximately 95% of the leukocytes in the first wave were PMNL whilst in the secondary wave measurements at 40 and 48 hr showed that 70% of the leukocytes were mononuclear cells.

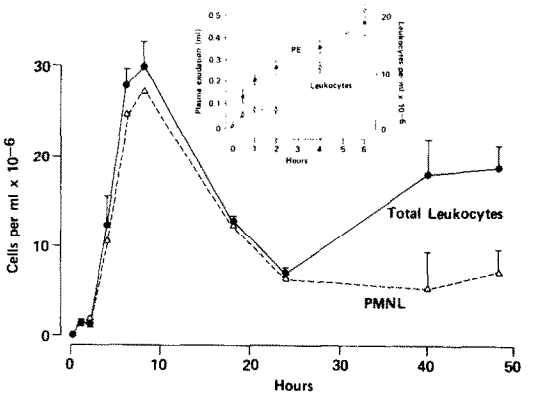

Fig. 1. Time course of the infiltration of leukocytes into 0.5% carrageenin-soaked sponges. Inset shows plasma exudation into the sponges monitored by the leakage of ^{125}I-HSA and its relationship to leukocyte infiltration. Data points are the means ± S.E. of 10–12 rats.

The leakage of plasma proteins into the sponges is also shown in Fig. 1 (inset). Approximately 75% of the total plasma exudation precedes leukocyte infiltration and this occurred most rapidly immediately following spong implantation. Plasma exudation was almost complete by 4 hr after sponge implantation.

Authentication of immunoreactive LTB_4

Authentication of the immunoreactive LTB_4 in the inflammatory exudate was carried out by reversed-phase HPLC fractionation. As shown in Fig. 2 a single peak of immunoreactive LTB_4 which co-eluted with authentic LTB_4 standard was identified. It is pertinent to note that the exudate LTB_4 content and profile described here (Figs 5 and 6) are very similar to those described by Simmons *et al.* [19] measured with a different antibody.

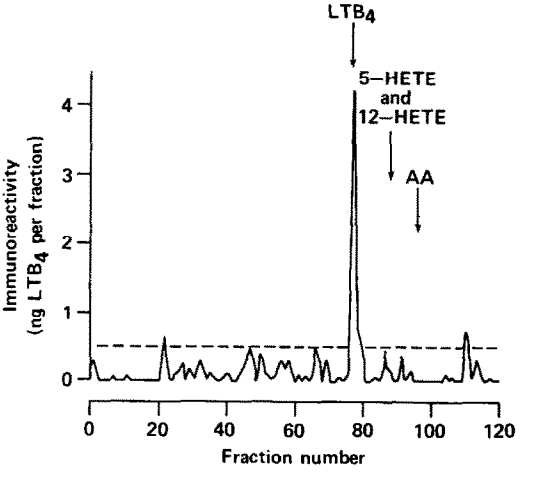

Fig. 2. Immunoreactive LTB_4 profile following fractionation of inflammatory exudate in 40% methanol by reversed-phase HPLC. Radiolabelled LTB_4, 12-HETE, 5-HETE and arachidonic acid were used to calibrate the column and 0.3 min fractions (500 μl) were collected, of which 20 μl were assayed for LTB_4. The dashed line shows the detection limit of the RIA.

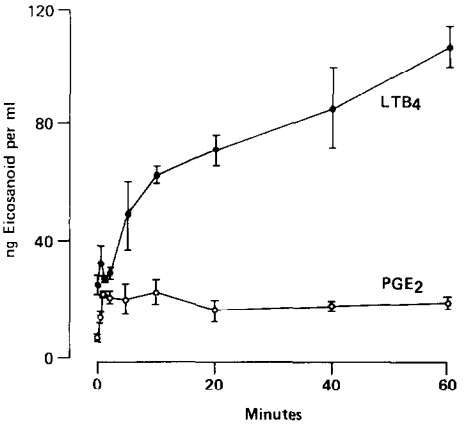

Fig. 3. Synthesis of LTB_4 (–●–) and PGE_2 (–○–) by exudate leukocytes stimulated with A23187. Incubates (250 μl) contained 12.0×10^6 leukocytes/ml of exudate. Each point is the mean ± S.E. of 4 observations.

Stimulation of eicosanoid biosynthesis by A23187

The generation of immunoreactive LTB_4 by A23187-stimulated exudate leukocytes was most rapid immediately after the addition of the ionophore with gradual decline through 60 min as shown in Fig. 3. In some experiments LTB_4 levels achieved a plateau by 30 min which was maintained through 60 min. The LTB_4 generated in this system was also shown to be authentic by reversed-phase HPLC fractionation of inflammatory exudate which had been stimulated for 30 min with A23187. A single peak of immunoreactivity was identified which co-eluted with authentic LTB_4 standard (data not shown). The time course of LTB_4 generation in this system differed from that described for human PMNL stimulated with A23187 in which LTB_4 is rapidly metabolised by w-oxidation [22–24].

We could find no evidence of w-oxidation by rat exudate leukocytes based on experiments in which [^{3}H] LTB_4 was added to exudate immediately prior to stimulation with A23187, followed by incubation at 37° for 2, 30 or 60 min at which times the cell-free incubates were fractionated by reversed-phase HPLC. Only single peaks of radioactivity were identified which co-eluted with [^{3}H] LTB_4 standard (data not shown).

Exudate leukocytes also generated PGE_2 when stimulated with A23187. This response was rapid and complete within 2 min as shown in Fig. 3.

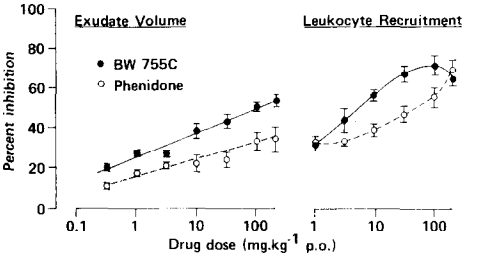

Fig. 4. Dose–response effect of BW755C and phenidone on the exudate volume and leukocyte content of 0.5% carrageenin-soaked subcutaneous sponge implants in rats. Compounds were dosed 1 hr prior to sponge implantation and inflammatory parameters were measured 4 hr after. Data is expressed as the percent inhibition of the control inflammatory parameters and are the means ± S.E. of from 4 to 6 experiments.

Effect of inhibitors of eicosanoid biosynthesis in vitro

The IC_{50} concentrations, calculated from dose–response experiments, for several lipoxygenase and cyclo-oxygenase inhibitors on the generation of LTB_4 and PGE_2 by A23187-stimulated exudate leukocytes are shown in Table 1. NDGA (IC_{50} 0.4 μM), quercetin (IC_{50} 0.4 μM) and phenidone (IC_{50} 0.3 μM) were approximately equipotent at inhibiting LTB_4 generation. The compounds, however, showed marked differences in their selectivity as reflected in the ratio of their IC_{50}s for PGE_2/LTB_4 which for quercetin, phenidone and NDGA were >250, 18.7 and 5.2 respectively. Nafazatrom which has been shown to inhibit LTB_4 formation in human PMNL [25] was only a weak inhibitor (IC_{50} 24.0 μM) in this system although inactive against cyclo-oxygenase at 100 μM. BW755C profiled as a mixed inhibitor being approx 3 times more selective against PGE_2 generation (IC_{50} 1.7 μM) than LTB_4 (IC_{50} 5.9 μM). The cyclo-oxygenase inhibitor indomethacin was a potent and selective inhibitor of PGE_2 generation (IC_{50} 1.3 μM) and actually stimulated LTB_4 production to maximal levels of approximately 160–180% of control levels at concentrations above 10^{-7} M.

Effect of compounds on inflammatory parameters and eicosanoid generation ex vivo

The mixed cyclo-oxygenase-lipoxygenase inhibitors BW755C and phenidone caused a dose-dependent inhibition of oedema and leukocyte content of the inflammatory exudate as shown in Fig. 4.

Table 1. Effect of compounds on the generation of eicosanoids by A23187-stimulated inflammatory exudate leukocytes

Compound	IC_{50} (μM) LTB_4 generation	IC_{50} (μM) PGE_2 generation	IC_{50} PGE_2/IC_{50} LTB_4
NDGA	0.4	2.1	5.2
Quercetin	0.4	>100	>250
Nafazatrom	24.0	>100	>4.0
Indomethacin	s*	1.3	—
BW755C	5.9	1.7	0.29
Phenidone	0.3	5.6	18.7

Values are the means from two experiments.
*s = stimulated.

Table 2. Effects of "mixed inhibitors" and NSAIDs on inflammatory parameters and *ex vivo* eicosanoid generation

Compound	Exudate* volume	Leukocyte migration	i-PGE_2 generation	i-LTB_4 generation
BW755C	3	5	17	16
Phenidone	60	60	170	13
Indomethacin	0.7	1.0	1.50	>10
Fluribprofen	1.0	0.8	0.13	>10

Approximate ED_{50} or ED_{30} * (mg per kg p.o.).

BW755C was approximately 12- and 20-fold more potent at inhibiting leukocyte infiltration and oedema respectively than phenidone (Table 2). BW755C and phenidone were approximately equipotent inhibitors of LTB_4 generation *ex vivo* but BW755C was 10-fold more potent at inhibiting PGE_2 generated *ex vivo* than phenidone (Table 2).

To try and shed further light on the anti-inflammatory mechanism of these mixed inhibitors, several other inhibitors of eicosanoid biosynthesis were evaluated. Nafazatrom (Bayer g6575), quercetin and NDGA, which inhibit the generation of LTB_4 by A23187-stimulated exudate leukocytes *in vitro* (Table 1) had no effect on the inflammatory oedema, leukocyte infiltration or on the generation of PGE_2 or LTB_4 by A23187-stimulated exudate leukocytes *ex vivo* when dosed at 100 mg/kg p.o. These agents were also inactive when dosed twice at 16 and 1 hr prior to sponge implantation.

Indomethacin and flurbiprofen were potent inhibitors of oedema formation (exudate volume), leukocyte infiltration and the generation of PGE_2 by A23187-stimulated exudate leukocytes *ex vivo* as shown in Table 2. These anti-inflammatory agents were highly selective inhibitors of PGE_2 generation and actually enhanced LTB_4 generation as reported elsewhere [18]. In the studies described here the effect of anti-inflammatory agents on the leukocyte infiltration into carrageenin-soaked sponges was calculated as the percent inhibition in the total number of leukocytes per sponge. This procedure takes into account the reduction in exudate volume caused by anti-inflammatory agents and therefore provides a better estimate of their efficacy against leukocyte recruitment than if the results were expressed as leukocytes/ml. As shown in Table 3, indomethacin and flurbiprofen when dosed at 1 mg/kg caused 23.2 and 25.2% inhibition in the number of leukocytes per ml of inflammatory exudate respectively. However, when the effect of these compounds on the exudate volume was taken into account, by calculating the total number of leukocytes per sponge, indomethacin and flurbiprofen caused 57.0 and 46.1% inhibition of leukocyte infiltration respectively.

Effect of mixed inhibitors on exudate leukocyte and eicosanoid content

In order to elucidate the contribution of LTB_4 to leukocyte recruitment in this model we investigated the effects of BW755C and phenidone on the leukocyte and eicosanoid content of the inflammatory exudate during the 8 hr period following sponge implantation. BW755C and phenidone when dosed at 100 mg/kg, 1 hr prior to sponge implantation, reduced the LTB_4 content of the exudate whilst phenidone had a more prolonged effect (Fig. 5). In addition BW755C reduced the exudate PGE_2 content to almost undetectable levels and inhibited the leukocyte content by greater than 50% throughout the 8 hr time course (Fig. 5). In marked contrast phenidone had negligible effect on the PGE_2 and leukocyte content of the exudate. BW755C and phenidone were also dosed to rats 2 hr after subcutaneous sponge implantation. As shown in Fig. 6, both agents inhibited the LTB_4 content of the exudate although apparently less than when the agents were dosed 1 hr prior to sponge implantation probably due to the high levels of LTB_4 already present in the exudate at the time of dosing. BW755C almost totally inhibited the exudate PGE_2 content and markedly the exudate leukocyte content during the 6 hr period following dosing as shown in Fig. 6. Phenidone, however, caused only a small reduction in the PGE_2 and leukocyte content of the exudate (Fig. 6).

Table 3. Inhibition of leukocyte infiltration by NSAIDs

Compound	N	Exudate volume (ml)	No. leukocytes per ml $\times 10^{-6}$	% Inhibition	No. leukocytes per sponge $\times 10^{-6}$	% Inhibition
Control	19	1.06 ± 0.04	12.09 ± 0.42	—	12.78 ± 0.63	—
Indomethacin	10	0.61 ± 0.08	9.28 ± 0.51	23.2*	5.49 ± 0.61	57.0*
Flurbiprofen	10	0.74 ± 0.23	9.04 ± 0.48	25.2*	6.88 ± 0.94	46.1*

Compounds were dosed (1 mg per kg p.o.) 1 hr prior to sponge implant. Exudates were recovered and inflammatory parameters measured 4 hr after sponge implantation.

Data are the means ± S.E.

* $P < 0.001$ compared to controls.

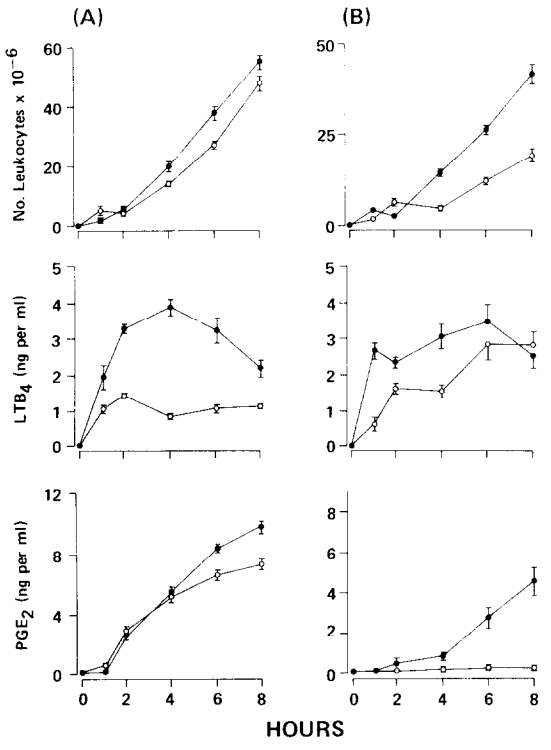

Fig. 5. Effect of (A) phenidone and (B) BW755C on leukocyte infiltration and eicosanoid content of the inflammatory exudate invoked by implantation of 0.5% carrageenin-soaked sponges in rats. Compounds were dosed (100 mg per kg p.o.) 1 hr prior to sponge implantation and the leucocyte, LTB_4 and PGE_2 content of the exudate measured at the times shown in the figure. Data points are the means ± S.E. of 9–10 control (●) or drug-treated (○) rats.

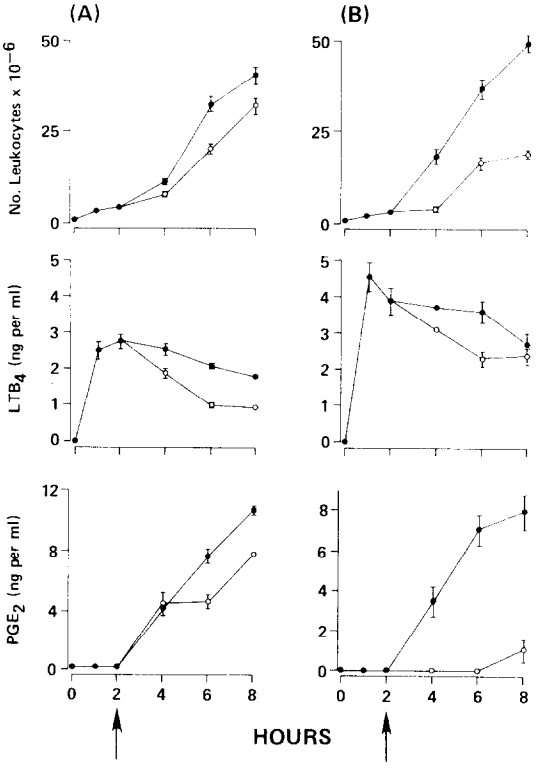

Fig. 6. Details of this experiment are as described in the legend to Fig. 5 except that compounds were dosed, as indicated by the arrows, 2 hr after sponge implantation.

Chemotactic effect of LTB_4 in vivo

Sterile polyester sponges soaked in various concentrations of LTB_4 in sterile saline were implanted subcutaneously into rats. The leukocyte content of the sponges determined 6 hr after implantation are shown in Fig. 7a. LTB_4 caused a small dose-related increase in the leukocyte content (95% PMNL) of the sponges which amounted to twice the saline control content ($2.3 \pm 0.05 \times 10^6$ cells/ml) at an LTB_4 concentration of 800 ng/ml. Sponges soaked in 0.5% carrageenin had a much more marked effect on leukocyte infiltration causing an 11-fold increase over control saline-soaked sponges (Fig. 7a).

Histological evaluation of sections incorporating the carrageenin-soaked sponge and its surrounding tissues, 4 hr after sponge implantation, revealed an envelope of PMNL surrounding the sponge (data not shown). Measurement of the leukocyte content of the sponge exudate therefore underestimates the total leukocyte content of the lesion and possibly the chemotactic potential of LTB_4. To compensate for such a possibility we measured histologically the leukocyte content of rat skin sites injected with a range of LTB_4 concentrations. As shown in Fig. 7b, 15 ng per site of LTB_4 was not chemotactic whereas concentrations ranging from 63 to 1000 ng per site caused an increase in the PMNL content of the tissues.

DISCUSSION

The objectives of these studies were to (1) evaluate the contribution of LTB_4 to leukocyte recruitment in the subcutaneous sponge implant model of acute inflammation in the rat, and (2) assess the model as a suitable test system for demonstrating the potential anti-inflammatory efficacy of inhibitors of LTB_4 synthesis.

LTB_4 is a potent chemotactic agent for leukocytes in several animal species [1–6] and man [7, 8]. The experiments reported here (Figs 5 and 6) clearly demonstrated that an increase in LTB_4 levels precede the infiltration of leukocytes into the inflammatory exudate. It is tempting to speculate therefore that LTB_4 may contribute to the subsequent recruitment of leukocytes into the exudate. This observation is not confined to carrageenin as stimulus or to the rat but appears to be generally associated with acute inflammatory reactions. Ford-Hutchinson *et al.* [12] observed a peak of LTB_4 which preceded the infiltration of leukocytes into zymosan-soaked sponges. We have also observed the same response in carrageenin-soaked sponges implanted into NZW rabbits. The response is not a non-specific reaction to the sponge since we and others have made similar observations in rat air pouches [26] challenged with a variety of stimuli.

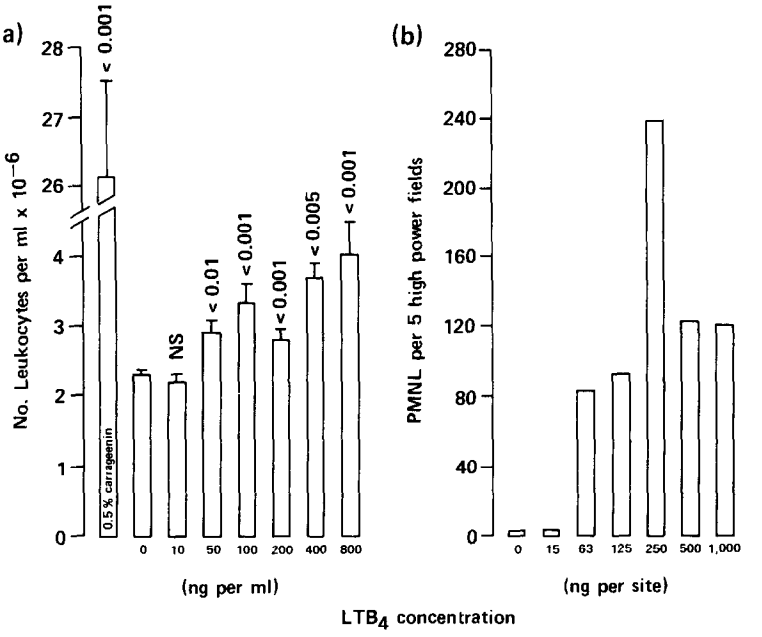

Fig. 7. (a) Infiltration of leukocytes into LTB_4-soaked polyester sponges implanted subcutaneously into rats. The leukocyte content of the sponges were measured 6 hr after implantation. Data bars are the means ± S.E. of 10–12 rats. The significance of the data was assessed using Student's *t*-test. (b) Infiltration of leukocytes into rat skin measured 2 hr after intradermal injections of LTB_4. The leukocyte content was assessed histologically by counting the number of PMNL in 5 highpower fields of 5 μ thick sections of skin stained with haematoxylin and eosin. Data bars are the means of duplicates.

Although the source of the LTB_4 is uncertain it is unlikely to be derived from plasma despite the close association between plasma exudation and the appearance of LTB_4 in the inflammatory exudate, since plasma contains less than 0.2 ng/ml of LTB_4 whilst exudate levels exceed this 20-fold. It has been demonstrated that when the migration of leukocytes is impaired by treating rats with the microtubule disrupting agent colchicine, both the PMNL and LTB_4 content of the exudate are reduced [11]. We have confirmed these observations in rats treated with colchicine and also vinblastine, an agent which depletes leukocytes, suggesting that leukocytes may be the source of the LTB_4. If this is the case, since LTB_4 appears in the exudate prior to leukocytes, LTB_4 must be released from the leukocytes prior to their transit into the sponge, perhaps when they first interact with the endothelial cells of the microvasculature.

If LTB_4 contributes to the recruitment of leukocytes into the sponge then inhibition of its biosynthesis should result in reduced leukocyte recruitment. Unfortunately, quercetin, nafazatrom and NDGA which inhibited the generation of LTB_4 *in vitro*, failed to inhibit LTB_4 generation *ex vivo* and not surprisingly had no effect on leukocyte recruitment. It is not known whether this is due to their poor absorption or pharmacokinetics. These observations serve as a caveat in drawing conclusions concerning the role of eicosanoids in inflammation based solely on the effect of drugs on inflammatory parameters. It is commonplace, as judged from the literature, to evaluate in animal models of inflammation, agents which inhibit eicosanoid synthesis *in vitro* without substantiating their biochemical efficacy *in vivo* or *ex vivo*. Conclusions concerning the anti-inflammatory mode of action of such inhibitors are invalid unless both biochemical and pharmacological efficacy are determined in the same experiments.

Salmon *et al.* [18] demonstrated that BW755C caused a dose-dependent inhibition in the exudate eicosanoid and leukocyte content. Our studies have confirmed their observations although in our hands BW755C was a more potent inhibitor (approximate ED_{50} 5 mg/kg) of leukocyte recruitment than they reported (approximate ED_{50} 72 mg/kg). This can be partly explained by the different methods used by Salmon *et al.* and ourselves to calculate the effect of the compound on leukocyte recruitment as discussed in the results section. Also in our studies leukocyte recruitment was measured 4 hr after sponge implantation rather than 6 hr as described by Salmon *et al.*

When rats were treated with an efficacious dose of BW755C (100 mg/kg) one hr prior to sponge implantation, a reduction in both the exudate LTB_4 and leukocyte content could be demonstrated for 6 hr following implantation. Phenidone which is at least equipotent with BW755C against LTB_4 generation (Table 2) was much less effective at inhibiting leukocyte recruitment. This observation suggests that BW755C exerts its effect on leukocyte recruitment by a mechanism which is independent of its inhibitory effect on LTB_4 synthesis.

Dose–response studies demonstrated BW755C effectively inhibited the generation of PGE_2 by exudate leukocytes *ex vivo*. The time course profile (Fig.

5) shows that BW755C (100 mg/kg) almost totally inhibited the PGE_2 content of the inflammatory exudate whereas phenidone had little effect suggesting that cyclo-oxygenase products may play a role in leukocyte recruitment in this model. Since the almost total inhibition of PGE_2 levels in the exudate did not coincide with a total reduction in the leukocyte content other factors, perhaps complement [27] must contribute to the recruitment process.

In the time course studies in which BW755C and phenidone were dosed to rats 2 hr after sponge implantation the exudate levels were already maximal at the time of dosing (Fig. 6). Therefore, we argued that if LTB_4 is the major chemotactic agent responsible for leukocyte recruitment in this model, a lipoxygenase inhibitor might be less effective or even inactive against leukocyte recruitment under these conditions. Again, BW755C almost totally inhibited PGE_2 levels whilst phenidone was much less effective. We conclude from these time course studies that the LTB_4 which precedes leukocyte infiltration into the sponge exudate does not mediate the leukocyte recruitment. A corollary of these observations is that the inhibitory effect of BW755C on leukocyte recruitment in this model is a function of its cyclo-oxygenase and not lipoxygenase inhibitory property, a point which was not emphasised by Salmon *et al.* [18]. Our data supports the view expressed by Steel *et al.* [28] that "inhibition of cell accumulation in this model by BW755C, dexamethasone, indomethacin and flurbiprofen is explicable in terms of a reduction in prostaglandins rather than LTB_4". This view is further supported by our observations that indomethacin and flurbiprofen which reduce the exudate PGE_2 content and PGE_2 generated by exudate leukocytes *ex vivo*, also inhibit the infiltration of leukocytes into the sponge. Inhibition of leukocyte recruitment by NSAIDs has been reported by others [29–31]. The mechanism by which NSAIDs inhibit leukocyte recruitment into inflammatory lesions in experimental animal models is unclear. The synergistic actions of the prostaglandins with LTB_4 and other mediators to promote plasma exudation is well established [9, 10]. Vasodilators such as the E-type prostaglandins also potentiate the accumulation of leukocytes into lesions by chemotactic agents [5, 32]. It is possible therefore that NSAIDs, through inhibition of vasodilatory prostaglandins, reduce the blood flow and therefore the delivery of leukocytes to the inflammatory lesion. Sponges which had been soaked in LTB_4 caused only a modest (2-fold) recruitment of leukocytes into the sponges in contrast to carrageenin (11-fold). Moreover, the threshold concentration of LTB_4 (50 ng/ml) required to cause such a modest increase in leukocyte recruitment was 10-fold higher than the maximal levels detected in the inflammatory exudate, an observation supported by histological assessment following intradermal LTB_4 administration. It would appear therefore that the levels of LTB_4 detected in the sponge (approx. 5 ng per ml) are insufficient to cause leukocyte recruitment.

Studies of the specific binding of radiolabelled LTB_4 to human peripheral PMNL have revealed specific receptor sites which appear to have a role in mediating the biological responses of leukocytes [33, 34]. In a recent publication Kreisel *et al.* [35] have demonstrated that rat PMNL lack a specific LTB_4 binding site which is present in human PMNL and is involved in the chemotactic response to LTB_4. This observation may explain the poor chemotactic response of the rat to LTB_4. The low level of leukocyte recruitment which we observed may be due to "unphysiological" levels of LTB_4.

In summary our results suggest that the cyclo-oxygenase products the prostaglandins make a major contribution to leukocyte recruitment in the rat sponge implant model of acute inflammation and that the lipoxygenase product LTB_4 makes little contribution. This model is therefore unsuitable for demonstrating the potential anti-inflammatory efficacy of 5-lipoxygenase inhibitors. Finally because LTB_4 is not a potent chemotactic agent in the rat there appears to be no advantage of investigating the role of LTB_4 in acute inflammation in this species.

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