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The effects of BW755C and other anti-inflammatory drugs on eicosanoid concentrations and leukocyte accumulation in experimentally-induced acute inflammation

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BW755C (3-amino-1-[m-trifluoromethyl)phenyl]-2-pyrazoline HCl) reduced the concentration of leukotriene B_4 (LTB_4), thromboxane B_2 (TXB_2) and prostaglandin E_2 (PGE_2) in exudate derived from the subcutaneous implantation in rats of 0·5% carrageenan-impregnated polyester sponges. Polymorphonuclear leukocyte (PMN) migration into the inflammatory exudate was also decreased. The inhibition of LTB_4 may, in part, account for the lower number of cells in the exudate since LTB_4 is a potent leukotactic agent. Inhibition of LTB_4-formation and cell migration by BW755C was dose-related, but the two dose-response curves were not parallel. Cell influx still occurred at doses of BW755C that completely inhibited formation of LTB_4: this indicates that, although LTB_4 may have a chemotactic role in-vivo, other factors must also contribute to cell migration into the inflammatory exudate. Treatment of rats with dexamethasone also caused a reduction in leukocytes and eicosanoids in the exudate. As with BW755C, there was a differential effect on PMN and LTB_4: dexamethasone (1 mg kg^{-1}) reduced PMN accumulation by 40% but LTB_4 formation was inhibited by 70%. Leukocyte accumulation was also inhibited by the non-steroidal anti-inflammatory drugs (NSAID's), indomethacin and flurbiprofen. These drugs reduced the concentration of both PGE_2 and TXB_2 in exudate but that of LTB_4 was unchanged. This suggests that reduction of PMN accumulation by indomethacin and flurbiprofen is mediated by a mechanism other than inhibition of LTB_4-synthesis. Aspirin also reduced the levels of PGE_2 and TXB_2 in the exudate but did not consistently affect PMN influx, thereby confirming that inhibition of cyclo-oxygenase does not reduce cell migration in inflammation.

Prostaglandins, particularly protaglandin E_2 (PGE_2), released during inflammation, contribute to the inflammatory response. Furthermore, their production is reduced by non-steroidal anti-inflammatory drugs (NSAID's), now recognized to be the mechanism of action of these compounds (see Ferreira & Vane 1974; Moncada & Vane 1979).

Polymorphonuclear leukocyte (PMN) infiltration, which is also a characteristic response of inflammation, is reduced only with doses of NSAID higher than those required to inhibit the cyclo-oxygenase (Higgs et al 1979), suggesting that prostaglandins do not play a primary role in the recruitment of cells. Anti-inflammatory steroids, in addition to reducing the concentration of cyclo-oxygenase products, do prevent PMN infiltration. These steroids do not block cyclo-oxygenase directly but inhibit the enzymic liberation of the prostaglandin precursor, arachidonic acid, from cell-membrane phospholipids (Flower & Blackwell 1979; Blackwell et al 1980).

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Arachidonic acid can also be metabolized by lipoxygenases to a series of hydroperoxy-eicosatetraenoic acids (HPETE's); one of these (5-HPETE) is of particular importance for it can be converted to leukotrienes (see Samuelsson 1981; Samuelsson et al 1980). The mono-hydroperoxy and hydroxy acids, but especially the dihydroxy acid, leukotriene B_4 (LTB_4), are chemokinetic and chemotactic for leukocytes in-vitro and in-vivo (Ford-Hutchinson et al 1980; Palmer et al 1980; Bhattacherjee et al 1981; Higgs et al 1981) and therefore synthesis of these products at sites of inflammation could be responsible for the influx of leukocytes. This hypothesis is strengthened by the observations that higher than normal concentrations of 12-hydroxy-eicosatetraenoic acid (12-HETE) and/or LTB_4 occur in involved psoriatic skin (Hammarstrom et al 1975; Brain et al 1982) and in synovial

fluid from patients with gout (Rae et al 1982) and rheumatoid arthritis (Klickstein et al 1980; Davidson et al 1982). Using a sensitive and specific radioimmunoassay (RIA) for LTB_4, we have demonstrated that LTB_4 is produced during experimentally-induced acute inflammation and that PMN's are the major source of this product (Simmons et al 1983). In the present study, we have evaluated the effects of BW755C, indomethacin, flurbiprofen, aspirin and dexamethasone on the concentrations of eicosanoids (LTB_4, PGE_2 and thromboxane B_2 (TXB_2)) and their relationship with leukocyte numbers in experimentally-induced inflammatory exudate.

MATERIALS AND METHODS

Materials

Polyester sponge (thickness 0·5 cm) and λ-carrageenan (Viscarin) were purchased from Transatlantic Plastics Ltd., Surbiton, Surrey and Marine Colloids, Springfield, New Jersey, U.S.A., respectively. Hanks' balanced salt solution was obtained from Wellcome Diagnostics, Dartford, Kent. HEPES buffer and silicic acid were from Sigma Chemical Co., St Louis, Missouri, U.S.A. The divalent cation ionophore, A23187, was purchased from Calbiochem, Bishop's Stortford, Herts. Indomethacin and dexamethasone sodium phosphate (Decadron) were obtained from Merck, Sharp and Dohme, Hoddesdon, Herts; aspirin was from The Wellcome Foundation, Beckenham, Kent and flurbiprofen from Boots, Nottingham. 3-Amino-1-[m-(trifluoromethyl)-phenyl]-2-pyrazoline hydrochloride (BW755C) was synthesized by Dr F. C. Copp, Wellcome Research Laboratories, Beckenham. $[^3H]LTB_4$ was kindly supplied by Dr D. Copsey, Amersham International, Amersham, Bucks. Analytical and h.p.l.c. grade solvents were purchased from BDH, Poole, Dorset and Rathburn Chemicals Ltd., Walkerburn, Peebles respectively.

Inflammatory exudates

Sterile polyester sponges soaked in 0·5% carrageenan (w/v sterile saline) were implanted subcutaneously in male Wistar rats (200–250 g) as previously described (Higgs et al 1976; Simmons et al 1983). Animals were killed by a blow to the head followed by cervical dislocation, 6 h after implantation. The sponges were removed and the exudates squeezed from the sponges into polypropylene tubes. An aliquot (40 μl) of the exudate was removed immediately for determination of the leukocyte count using a Model ZBI Coulter counter (Coulter Associates). A further portion (500 μl) of the exudate was used to assess the capacity of the inflammatory cells to synthesize LTB_4 ex-vivo (see below). The remainder of the exudate was centrifuged at 12 000*g* for 30 s in a micro-centrifuge (Type 320; Burkard Scientific) to precipitate cells and debris. The supernatants were immediately assayed or stored frozen until analysis could be completed.

Formation of LTB_4 by inflammatory cells ex-vivo

After 30 min pre-incubation of exudate (500 μl) at 37 °C, A23187 (5 μg in 10 μl HEPES buffered Hanks' balanced salt solution, pH 7·4: HHBS) was added and incubation continued for 5 min. The reaction was terminated by centrifugation at 12 000*g* for 30 s; the supernatant was removed and assayed for LTB_4.

Effect of drugs

(a) *In-vivo*. Dexamethasone was administered by intraperitoneal (i.p.) injection 1 h before sponge implantation to achieve a maximum effect at 6 h (Church & Miller 1978); pre-dosing is required for synthesis of an antiphospholipase polypeptide (Blackwell et al 1980). Other drugs were given by single bolus i.p. injection immediately after sponge implantation. Each drug treatment was administered to at least five animals on two separate occasions and data compared with those obtained after administration of the relevant vehicle. Aspirin and flurbiprofen were administered in sodium bicarbonate solution (5% w/v); indomethacin was given in 50 mM Tris buffer (pH 7·4); BW755C hydrochloride was dissolved in water and dexamethasone (Decadron) was diluted with 0·9% NaCl (saline). The responses to the vehicles alone were not significantly different from each other or from untreated animals.

(b) *Ex-vivo*. The formation of LTB_4 by A23187-stimulated cells in the inflammatory exudate from drug-treated animals was monitored as described above ('Formation of LTB_4 by inflammatory cells ex-vivo').

(c) *In-vitro*. An aliquot (500 μl) of pooled inflammatory exudate from control rats was incubated at 37 °C for at least 30 min before addition of drug under test (10 μl giving a final concentration of 10^{-9}–10^{-4} M). After a further 15 min at 37 °C, A23187 (5 μg in 10 μl) was added and incubation continued for 5 min. The mixture was then processed as described above for formation of LTB_4 by inflammatory cells ex-vivo.

Assay of eicosanoids

Leukotriene B_4 in the exudate supernatant, diluted 1:5 in assay buffer, was measured by specific RIA (Salmon et al 1982a) without prior extraction or

chromatography. The concentrations of PGE_2 and TXB_2 in the exudate (diluted 1:10) were similarly determined by RIA; the specificity of these antisera and details of the procedure were reported by Salmon (1978).

To confirm the identity of immunoreactive LTB_4, combined cell-free exudates from groups of 10 animals treated with vehicle, indomethacin ($10\,mg\,kg^{-1}$) or BW755C ($100\,mg\,kg^{-1}$) were subjected to extraction, silicic acid and high pressure liquid chromatography (h.p.l.c.) before RIA as described by Salmon et al (1982a).

Statistics. Results were calculated as mean ± standard error of the mean. The significance of difference between data was assessed using Student's unpaired *t*-test.

RESULTS

Inflammatory exudate obtained 6 h after sponge implantation from control groups of rats contained $17{\cdot}42 \pm 0{\cdot}61 \times 10^6$ PMN ml^{-1} (n = 70). The concentrations of PGE_2, TXB_2 and LTB_4 in the exudate were 16·15 ± 1·00 (n = 70), 18·24 ± 1·12 (n = 65) and 5·94 ± 0·34 (n = 70) ng ml^{-1}, respectively. The concentrations of all three eicosanoids in inflammatory exudate were decreased in a dose-related manner by BW755C (10–200 mg kg^{-1}) (Fig. 1). It was a more effective inhibitor of the cyclo-oxygenase products, PGE_2 and TXB_2, than the lipoxygenase product, LTB_4 (Table 1). BW755C did inhibit the accumulation of cells in the exudate but was less active in this respect and the dose-response curve

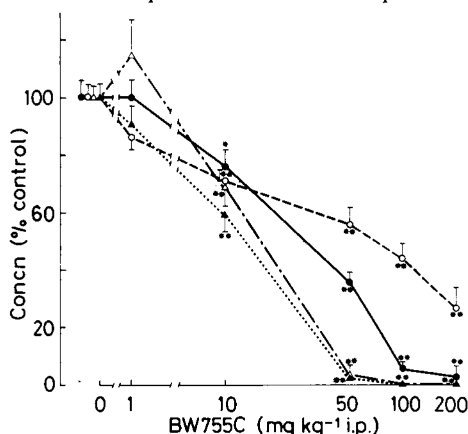

FIG. 1. Effect of BW755C on leukocyte count and eicosanoid concentration in inflammatory exudate obtained from s.c. implantation in rats of sponges impregnated with 0·5% carrageenan. ○– – –○ Leukocyte number; ●——● LTB_4; △—·—△ PGE_2; ▲– – –▲ TXB_2. Each point is the mean ± s.e. (n = 10).

Table 1. Inhibition of leukocyte count and eicosanoid synthesis by anti-inflammatory drugs.

Compound	Approximate IC50 (mg kg^{-1} i.p.) PMN	LTB_4	PGE_2	TXB_2
BW755C	72	28	16	13
Indomethacin	3·2	>10†	0·4	0·3
Aspirin	>500*	>500†	120	23
Flurbiprofen	0·8	>10†	0·1	0·05
Dexamethasone	>10*	0·7	0·7	0·4

* 50% inhibition of PMN migration was not achieve at the doses tested; maximum inhibition (28% and 42%) was obtained after 50 and 1 mg kg^{-1} of aspirin and dexamethasone, respectively.

† LTB_4-synthesis was not inhibited by these drugs (see Figs 4 and 5).

was not parallel to that obtained for inhibition of either cyclo-oxygenase or lipoxygenase products (Fig. 1; Table 1); approximately 55% inhibition of the leukocyte count occurred after 100 mg kg^{-1} BW755C, a dose at which eicosanoid synthesis was almost completely abolished (see Fig. 1). BW755C also inhibited the synthesis of LTB_4 by cells stimulated with A23187 ex-vivo, at doses that reduced the concentration of LTB_4 in the exudate (Fig. 2); the stimulated synthesis of LTB_4 in control exudate was 29·85 ± 3·47 ng ml^{-1} (n = 45).

The ex-vivo and in-vitro dose-response curves for inhibition of A23187-induced LTB_4 synthesis by BW755C (Figs 2 and 3, respectively) suggest that the concentration causing a 50% reduction of LTB_4 formation (approx. 5×10^{-6} M) can be achieved by a dose of 20 mg kg^{-1} i.p.

Indomethacin caused a dose-dependent reduction in the concentration of PGE_2 and TXB_2 in the inflammatory exudate (approximate IC50 0·4 mg kg^{-1}) but the concentration of LTB_4 was not decreased (Fig. 4). Higher doses of indomethacin also lowered the cell count in the exudate (approxi-

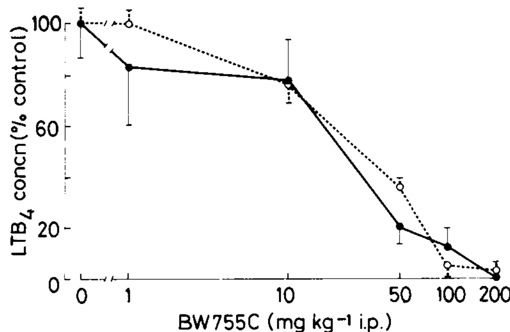

FIG. 2. Effect of BW755C on LTB_4 concentration in inflammatory exudate (○– – –○) and on LTB_4 synthesized ex-vivo in exudate stimulated with A23187 (●——●). Each point is the mean ± s.e. (n = 10).

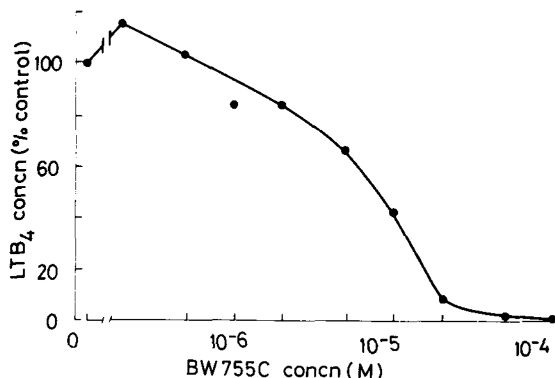

FIG. 3. Effect of BW755C in-vitro on LTB_4-synthesis in inflammatory exudate stimulated with A23187. Each point is the mean of duplicates.

mate IC50 3 mg kg^{-1}). This profile of activity was shared by another NSAID, flurbiprofen (Table 1) which was the more potent. Aspirin caused a greater inhibition of TXB_2 (IC50 23 mg kg^{-1}) than PGE_2 (IC50 120 mg kg^{-1}) in the exudate and was less effective than either indomethacin or flurbiprofen at reducing the leukocyte count, in fact at higher doses (100–500 mg kg^{-1}) there was a tendency for the cells and LTB_4 to increase over control values (Table 1; Fig. 5); significant ($P < 0.01$) inhibition (approximately 30%) of cells occurred only with 50 mg kg^{-1} aspirin and the IC50 was in excess of 500 mg kg^{-1}.

Dexamethasone had an effect similar to that of BW755C on leukocyte migration and eicosanoid production (Fig. 6) although complete inhibition of eicosanoid synthesis was not achieved even at the high dose of 10 mg kg^{-1} (not shown). The inhibition of eicosanoid synthesis caused by dexamethasone was greater than that of leukocyte accumulation; after 1 mg kg^{-1} the inhibition of LTB_4 and TXB_2 synthesis was 70 and 80% respectively whereas the PMN count was only reduced by 40%.

Immunoreactive LTB_4 in the sponge exudate from control and indomethacin-treated (10 mg kg^{-1}) rats had an identical h.p.l.c. mobility to that of authentic LTB_4. No immunoreactive material was detected in exudates from rats treated with BW755C (100 mg kg^{-1}).

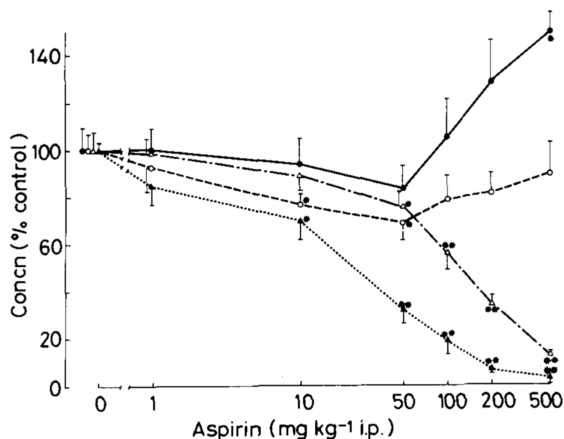

FIG. 5. Effect of aspirin on leukocyte count and eicosanoid concentration in inflammatory exudate obtained from s.c. implantation in rats of sponges impregnated with 0·5% carrageenan. ○- - -○ Leukocyte number; ●——● LTB_4; △—·—△ PGE_2; ▲- - -▲ TXB_2. Each point is the mean ± s.e. (n = 10).

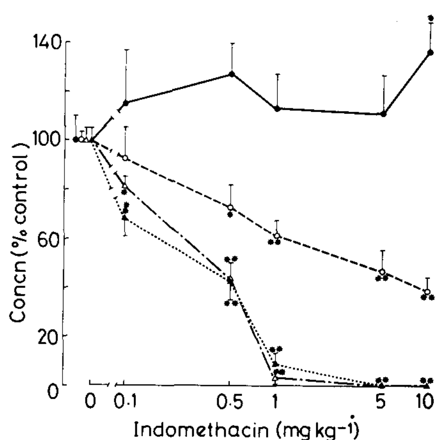

FIG. 4. Effect of indomethacin on leukocyte count and eicosanoid concentration in inflammatory exudate obtained from s.c. implantation in rats of sponges impregnated with 0·5% carrageenan. ○- - -○ Leukocyte number; ●——● LTB_4; △—·—△ PGE_2; ▲- - -▲ TXB_2. Each point is the mean ± s.e. (n = 10).

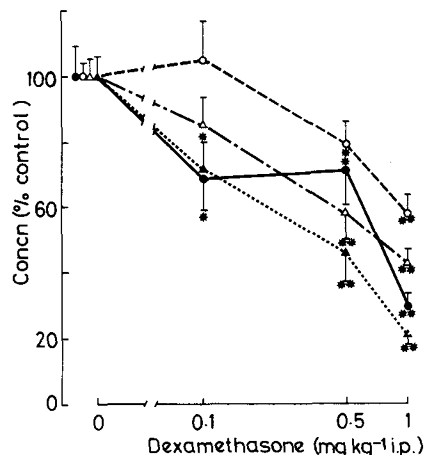

FIG. 6. Effect of dexamethasone on leukocyte count and eicosanoid concentration in inflammatory exudate obtained from s.c. implantation in rats of sponges impregnated with 0·5% carrageenan. ○- - -○ Leukocyte number; ●——● LTB_4; △—·—△ PGE_2; ▲- - -▲ TXB_2. Each point is the mean ± s.e. (n = 10).

DISCUSSION

BW755C inhibits metabolism of arachidonic acid via the 5-lipoxygenase in-vitro (Randall et al 1980; Radmark et al 1980) and this has been confirmed in the present study. Our data also demonstrate that administration of BW755C to rats reduces the concentration of the 5-lipoxygenase product, LTB_4, in inflammatory exudate. Also, BW755C lowered the concentration of the cyclo-oxygenase products, PGE_2 and TXB_2, in the exudate. Inhibition of the synthesis of LTB_4, a potent leukotactic agent, may cause, in part, the decrease in cell count in the exudate after administration of BW755C. However, other factors are also involved because cell influx was still observed when LTB_4 synthesis was abolished by BW755C.

Indomethacin and the other NSAID's tested (flurbiprofen and aspirin) reduced the exudate concentration of PGE_2 and TXB_2 but not LTB_4. Clearly, the inhibition of PMN influx by indomethacin and flurbiprofen observed by others (Ford-Hutchinson et al 1977; Higgs et al 1979, 1980), and confirmed in the present study, is not due to a reduction of the concentration of LTB_4 in the inflammatory exudate. The failure of indomethacin and flurbiprofen to reduce the concentration of LTB_4 even at doses that lower cell numbers is paradoxical. As the major source of LTB_4 in the exudate is the PMN (Simmons et al 1983), it would be expected that a reduction in cell number would lead to a lower concentration of LTB_4. This paradox may be explained by the observed inhibition of cyclo-oxygenase activity allowing more arachidonic acid to be metabolized via the lipoxygenase pathway and thereby increasing the formation of LTB_4. Alternatively, prostaglandin E_2 and prostacyclin, which is also produced in this inflammatory model and follows the same time course as PGE_2 (Higgs & Salmon 1979), increase cyclic-AMP levels in PMN and this could reduce the formation of LTB_4 (Claesson et al 1981) and thus, the removal of these cyclo-oxygenase products by treatment with NSAID could lead to increased synthesis of LTB_4. This latter explanation is supported by the observation that the LTB_4-concentration is increased after administration of aspirin only at doses at which it inhibited PGE_2 production. Increased synthesis of LTB_4 was not observed at lower doses of aspirin which blocked TXB_2-formation. Since LTB_4 and TXB_2 are formed in the same cell-type, PMN, this implies that diversion of substrate is not a valid explanation.

Polymorphonuclear leukocytes metabolize LTB_4 (Hansson et al 1981; Salmon et al 1982b) and, thus, a lower cell count could reduce the rate of catabolism; this, together with an increased synthesis of LTB_4 due to inhibition of PGE_2 and prostacyclin, could explain the maintenance of high levels of LTB_4 in the exudate in spite of a decrease in cell numbers.

Aspirin was less effective than either indomethacin or flurbiprofen at lowering the cell count and the effect only reached significance after 10 ($P < 0{\cdot}02$) and 50 mg kg^{-1} ($P < 0{\cdot}01$); higher doses were less effective. Interestingly, at high doses of aspirin (100–500 mg kg^{-1}) both the cell count and the levels of LTB_4 increased; the LTB_4-concentration was significantly greater than control after 500 mg kg^{-1}. These effects occurred at doses that inhibited synthesis of PGE_2 (and prostacyclin; data not shown) suggesting, as discussed previously for indomethacin, that PGE_2 and prostacyclin influence LTB_4-formation and, probably, cell influx into the exudate. Therefore, inhibition of PGE_2 and prostacyclin-synthesis causes an increase of LTB_4-formation and, possibly, cell migration. Although aspirin inhibited both cyclo-oxygenase products measured, it preferentially lowered TXB_2 whereas indomethacin and flurbiprofen caused similar inhibition of PGE_2 and TXB_2. In a previous study (Higgs et al 1983), we did not observe this differential effect of aspirin on the inhibition of cyclo-oxygenase products. However, some changes in methodology were introduced in the present study for reasons discussed by Simmons et al (1983) and this produced different data. In particular, with the methods employed in the present investigation, almost all the PGE_2 in the exudate was derived from tissues (Simmons et al 1983) whereas a large proportion of the PGE_2 detected in the previous study was synthesized by the PMN (Higgs et al 1983). Therefore, the differential effect of aspirin observed in this investigation suggests that the cyclo-oxygenase of the PMN is more easily inhibited than that in the tissues which generate PGE_2, possibly because the enzyme in various cell-types exhibits different sensitivities to inhibition by aspirin. Alternatively, this observation could reflect the pharmacokinetic profile of aspirin; rapid metabolism of aspirin limits its distribution.

The anti-inflammatory steroids block the release of free arachidonic acid from cell-membrane phospholipids by inducing the synthesis and release of a peptide that inhibits phospholipase A_2 (Flower & Blackwell 1979; Blackwell et al 1980) and this accounts for the reduced concentration of LTB_4 as well as PGE_2 and TXB_2 observed in the present study. However, dexamethasone (up to 10 mg kg^{-1}) did not produce 100% inhibition of either cell

migration or eicosanoid synthesis. The effect of steroids in-vitro and in-vivo is both dose- and time-dependent (Church & Miller 1978; Blackwell et al 1982) and, therefore, it is possible that the dosing regimen employed in this investigation was not optimal. As with BW755C, we are unable to conclude that the reduction of cell influx is due to lower LTB_4-synthesis because the depression of LTB_4 was more marked than the decrease in cell count.

In conclusion, indomethacin, flurbiprofen, dexamethasone, BW755C and aspirin, the latter being the least active, inhibit cell migration in the animal model of acute inflammation described. This action is not related to inhibition of prostaglandin synthesis since aspirin does not inhibit cell influx into the inflammatory exudate at doses that block the formation of PGE_2. The reduction of cell migration by BW755C and dexamethasone could be ascribed, at least in part, to the inhibition of LTB_4-synthesis. However, this argument is not valid in the case of indomethacin and flurbiprofen which decrease cell numbers without reducing the concentration of LTB_4. Factor(s), other than LTB_4, are certainly involved in the cellular response; for example, it has been demonstrated that the complement pathway and chemotactic peptides are probably involved in the mediation of cell accumulation in inflammation (Ward & Zvaifler 1971; Lynn et al 1978; Wright & Gallin 1978; Sedgwick et al 1982). Thus, indomethacin, flurbiprofen and aspirin could inhibit cell migration via a different mechanism, the nature of which remains to be investigated.

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