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Inhibition of leucotriene B_4 synthesis by BW 755c does not reduce polymorphonuclear leucocyte (PMNL) accumulation induced by monosodium urate crystals

M J FORREST, V ZAMMIT, AND P M BROOKS

From the Department of Rheumatology, Royal North Shore Hospital of Sydney, St Leonards, NSW 2065, Australia

SUMMARY Monosodium urate (MSU) crystals induce an inflammatory response when injected into the rat subcutaneous air pouch, which is characterised by polymorphonuclear leucocyte (PMNL) accumulation and plasma leakage. The arachidonic acid metabolites leucotriene B_4 (LTB_4), prostaglandin E_2 (PGE_2), 6-oxo-prostaglandin $F_{1\alpha}$, (6-oxo-$PGF_{1\alpha}$), and thromboxane B_2 (TXB_2) are found in increased concentrations in MSU induced exudates compared with animals injected with phosphate buffered saline (PBS). Pretreatment of animals with BW 755c significantly reduced the concentration of both lipoxygenase and cyclo-oxygenase derived arachidonic acid metabolites. Although BW 755c reduced MSU crystal induced plasma leakage, it did not affect PMNL accumulation. Pretreatment of animals with indomethacin selectively inhibited the generation of cyclo-oxygenase derived arachidonic acid metabolites and reduced MSU crystal induced plasma leakage but had no effect on PMNL accumulation. The inhibition of plasma leakage by either BW 755c or indomethacin was reversed by prostaglandin E_2 (1 μg/ml), which itself produced a significant increase in plasma leakage. The injection of purified LTB_4 (4 ng/ml or 40 ng/ml) did not induce either plasma leakage or PMNL accumulation within the air pouch. These data suggest that although MSU crystals stimulate LTB_4 production, LTB_4 is not the mediator of MSU crystal induced PMNL accumulation. Cyclo-oxygenase products of arachidonic acid metabolism (e.g., PGE_2), however, appear to play a part in MSU crystal induced plasma leakage.

Key words: anti-inflammatory, air pouch, gout, inflammation, plasma leakage.

There is a good deal of evidence to suggest that leucotriene B_4 (LTB_4) may be important in the pathogenesis of gout. An increased concentration of LTB_4 has been detected in the synovial fluid of patients with gout[1] and in the supernatants of polymorphonuclear leucocytes (PMNLs) incubated in vitro with monosodium urate (MSU) crystals.[2] LTB_4 is chemotactic both in vivo[3-5] and in vitro[6 7] for PMNLs and increases vascular permeability.[8 9] We have shown that the injection of MSU crystals into the rat subcutaneous air pouch initiates an acute inflammatory response characterised by PMNL accumulation, increased plasma leakage, and generation of LTB_4.[10]

The aim of this study was to elucidate whether or not LTB_4 generated in response to MSU crystals was mediating MSU induced PMNL accumulation and plasma leakage in the rat air pouch. To answer this question we investigated the effects of BW 755c, a combined lipoxygenase and cyclo-oxygenase inhibitor of arachidonic acid metabolism,[11] and indomethacin, a cyclo-oxygenase inhibitor, on MSU induced inflammation.

Although BW 755c inhibited LTB_4 production, the drug had no effect on MSU induced PMNL accumulation. Both BW 755c and indomethacin

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Correspondence to Dr M J Forrest, Division of Immunology Research, Merck Sharp and Dohme Research Laboratories, PO Box 2000, Rahway, New Jersey 07065.0900, USA.

inhibited MSU induced plasma leakage, suggesting the involvement of a cyclo-oxygenase product of arachidonic acid metabolism. This contention was supported by the observation that PGE_2 reversed the effects of both BW 755c and indomethacin on MSU induced plasma leakage.

Further doubt as to the role of LTB_4 in MSU crystal induced inflammation in the rat air pouch was cast by finding that purified LTB_4 failed to induce inflammatory changes in this model.

Materials and methods

FORMATION OF THE AIR POUCH

Air pouches were formed by methods described previously.[10] Briefly, male Wistar rats (Gore Hill Research Laboratories, St Leonards, NSW) weighing between 150 g and 180 g were used in all experiments. About 15–20 ml of sterile air was injected subcutaneously. This procedure was repeated three days later, and animals were used one week after the first injection of air.

MSU INDUCED INFLAMMATION

Rats with air pouches were injected intravenously with Evans blue dye (Sigma Chemical Company, MO, USA) (2·5% w/v; 2·0 ml/kg) to monitor plasma albumin leakage into the air pouch, together with mepyramine (5·0 mg/kg; Hamilton Labs Pty Ltd, South Australia), and methysergide (5·0 mg/kg; Sandoz Australia Pty Ltd) to antagonise the effects of endogenously released amines.[12] They were then injected with 10 ml of either phosphate buffered saline (PBS) or MSU crystals (10 mg/ml) suspended in PBS. MSU crystals were prepared according to the method of Denko and Whitehouse.[13] Four hours later the animals were killed with an overdose of pentobarbitone, and a 1 ml sample of blood was collected by cardiac puncture into edetic acid (10 mmol/l final concentration). The air pouch was opened with scissors and the exudate removed with a plastic pipette into a plastic tube containing 400 µl of 0·25 M edetic acid. The volume of exudate was recorded, a 100 µl sample removed for a cell count, and the remainder immediately centrifuged at 8000 *g* for one minute. The supernatant was then frozen at −20°C until further processing.

MEASUREMENT OF PLASMA LEAKAGE

The total amount of plasma in the air pouch was calculated from the ratio of the optical absorbance of the exudate to the absorbance of the plasma at 620 nm (λ_{max} for Evans blue dye). This figure was multiplied by the volume of exudate in the pouch to give the total microlitres of plasma in the air pouch.

MEASUREMENT OF CELL ACCUMULATION

Exudates were diluted 1:20 with gentian violet and the number and type of cells present counted by light microscopy.

EXTRACTION OF ARACHIDONIC ACID METABOLITES

Exudate (1 ml) was applied to a C-18 solid phase extraction column (Baker Chemical Company, NJ, USA), which was washed successively with 20 ml water, 2 ml hexane, and 2 ml methylene chloride. The column was then eluted with 2 ml methanol which was dried under a stream of nitrogen and, finally, resuspended in the radioimmunoassay buffer.

MEASUREMENT OF ARACHIDONIC ACID METABOLITES

Leucotriene B_4 was measured by radioimmunoassay using the Amersham radioimmunoassay kit (Amersham, UK). Prostaglandin E_2, 6-oxo-prostaglandin $F_{1\alpha}$, and thromboxane B_2 were measured by radioimmunoassay kits supplied by New England Nuclear (Boston, USA).

DRUG TREATMENT

Indomethacin (Merck Sharp and Dohme Australia Pty Ltd) was administered intravenously (2 mg/kg) and BW 775c (Wellcome Australia Pty Ltd) orally (50 mg/kg) immediately before the injection of MSU crystals or PBS into the air pouch. Prostaglandin E_2 (100 ng/ml or 1000 ng/ml) was administered mixed with the MSU crystals or PBS for injection into the air pouch.

PREPARATION OF LTB_4

Rat PMNLs were obtained from the peritoneal cavities of animals four hours after the injection of 20 ml of a 5% peptone solution in PBS. The cells were washed in Ca^{++} and Mg^{++} free Hanks's buffered salt solution (HBSS), then resuspended in complete HBSS containing 0·25% bovine serum albumin. The cells were incubated with arachidonic acid (10 µg/ml) and calcium ionophore A23187 (1 µg/ml) at 37°C for 15 minutes. The cell suspension was immediately centrifuged at 2500 *g* for 15 minutes and the LTB_4 extracted as described earlier. This crude extract was further purified by high performance liquid chromatography using an Altex Ultrasil ODS column (25×0·46 cm), as described previously.[10] LTB_4 was identified and measured by comparison with the elution time and peak height of synthetic LTB_4.

DATA ANALYSIS

Values reported are the mean (SEM) with n values reported as necessary. Comparison between groups was by Student's *t* test.

Results

EFFECT OF BW 755C AND INDOMETHACIN ON THE GENERATION OF ARACHIDONIC ACID METABOLITES

Injection of MSU crystals into the air pouch produced a significant ($p<0.001$) increase in the concentration of LTB_4 in the inflammatory exudate compared with PBS injected controls (Table 1). Prior treatment of animals with BW 755c significantly ($p<0.001$) reduced the concentration of LTB_4, though the amount present was still greater than that found in control animals. Indomethacin did not affect the amount of LTB_4 present.

The cyclo-oxygenase products of arachidonic acid metabolism, PGE_2, 6-oxo-$PGF_{1\alpha}$, and TXB_2, were all significantly raised in MSU injected animals compared with PBS injected controls (Table 2). Both BW 755c and indomethacin significantly reduced all the products. Indomethacin consistently inhibited the generation of cyclo-oxygenase products to a greater extent than did BW 755c.

Table 1 *Leucotriene B_4 (LTB_4) was measured in the inflammatory exudates of rats injected with phosphate buffered saline (PBS) or monosodium urate (MSU) crystals and the effect of BW 755c (50 mg/kg) and indomethacin (2 mg/kg) on LTB_4 generation determined**

Treatment	*Leucotriene B_4 (pg/ml)*
PBS	584 (98)
MSU	2429 (227)
MSU+BW 755c	1001 (68)
MSU+indomethacin	2917 (468)

*Results are the mean (SEM) values from six animals.

Table 2 *Concentrations of prostaglandin E_2 (PGE_2), 6-oxo-prostaglandin $F_{1\alpha}$ (6-oxo-$PGF_{1\alpha}$), and thromboxane B_2 (TXB_2) were measured in the inflammatory exudates of animals injected with phosphate buffered saline (PBS) or monosodium urate (MSU) crystals. The effect of BW 755c (50 mg/kg) and indomethacin (2 mg/kg) on the generation of these arachidonic acid metabolites was also determined**

Treatment (n)	*PGE_2 (pg/ml)*	*6-oxo-$PGF_{1\alpha}$ (pg/ml)*	*TXB_2 (pg/ml)*
PBS (8)	121 (23)	423 (49)	118 (14)
MSU (7)	12 353 (3995)	34 443 (9749)	16 717 (4340)
MSU+BW 775c (6)	2 521 (852)	1 893 (100)	710 (118)
MSU+indomethacin (8)	101 (21)	333 (240)	128 (24)

*Data are presented as the mean (SEM) of replicate determinations shown as n values in parentheses.

EFFECT OF BW 755C AND INDOMETHACIN ON MSU INDUCED PMNL ACCUMULATION

MSU crystals induced a significant ($p<0.001$) increase in PMNL accumulation compared with PBS injected controls (Fig. 1). Neither BW 755c nor indomethacin reduced the number of PMNLs in these inflammatory exudates.

EFFECT OF BW 755C, INDOMETHACIN, AND PGE_2 ON MSU INDUCED PLASMA LEAKAGE

The volume of exudate fluid recovered from PBS injected animals (6·08 (SEM 0·17) ml; n=22) was not significantly increased in animals injected with MSU crystals (6·29 (0·16) ml; n=28). Pretreatment of animals with indomethacin or BW 755c did not alter the volume of exudate fluid (5·93 (0·22) ml; n=13 and 6·26 (0·23) ml; n=16 respectively). Furthermore, the addition of prostaglandin E_2 to any of the treatment groups did not alter the volume

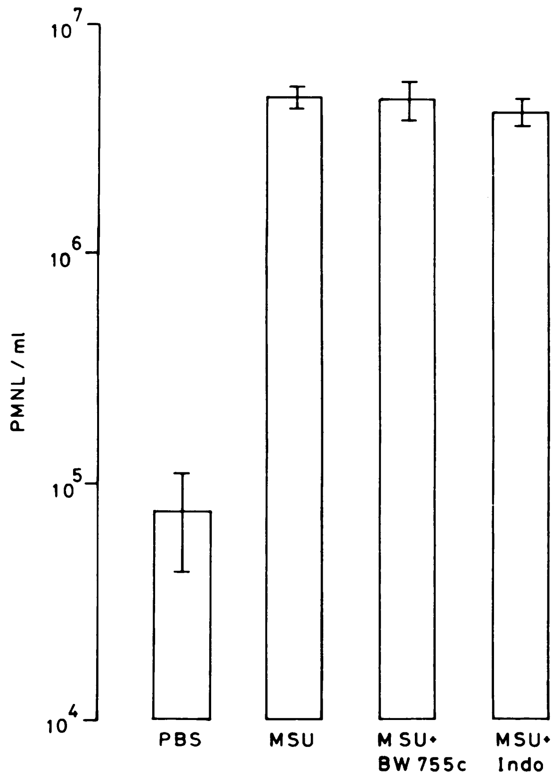

Fig. 1 *PMNL accumulation was determined in inflammatory exudates recovered from animals injected with phosphate buffered saline (PBS, n=22), monosodium urate (MSU) crystals (n=28), or MSU crystals in animals pretreated with indomethacin (2 mg/kg, n=13) or BW 755c (50 mg/kg, n=16). Each bar represents the mean and vertical lines represent the SEM.*

of exudate recovered from the air pouch. Significant differences in the concentration of albumin bound Evans blue dye were found between treatment groups, which reflect differences in plasma leakage into the air pouch.

Increased plasma leakage induced by MSU crystals was significantly inhibited by both BW 755c ($p<0{\cdot}001$) and indomethacin ($p<0{\cdot}001$) (Fig. 2). This inhibition was reversed by administration of PGE_2 at 1000 ng/ml but not at 100 ng/ml when injected with the crystals into the air pouch. The injection of prostaglandin E_2 alone at 1000 ng/ml into the air pouch significantly ($p<0{\cdot}001$) increased plasma leakage above that of PBS injected animals. Prostaglandin E_2 at a concentration of 100 ng/ml was without effect on plasma leakage.

INFLAMMATORY RESPONSE TO LTB_4 IN THE AIR POUCH

Injection of purified LTB_4 at a concentration of 4 ng/ml, equivalent to the level previously detected in MSU crystal induced exudate fluid,[10] or at 40 ng/ml did not produce a significant increase in PMNL accumulation or plasma leakage compared with that of PBS injected animals (Table 3).

Table 3 *Plasma protein leakage and polymorphonuclear leukocyte (PMNL) accumulation were measured in inflammatory exudates in response to phosphate buffered saline (PBS), monosodium urate (MSU) crystals, and leucotriene B_4 (LTB_4) (4 ng/ml and 40 ng/ml)**

Treatment	*Plasma (μl)*	*PMNLs/ml*
PBS (22)	306 (12)	$7{\cdot}6\ (3{\cdot}4)\times10^4$
MSU (28)	625 (23)	$4{\cdot}8\ (0{\cdot}5)\times10^6$
LTB_4: 4 ng/ml (8)	239 (35)	$4{\cdot}2\ (0{\cdot}9)\times10^5$
LTB_4: 40 ng/ml (8)	282 (42)	$4{\cdot}3\ (1{\cdot}8)\times10^5$

*Data are presented as the mean (SEM) of replicate determinations shown as n values in parentheses.

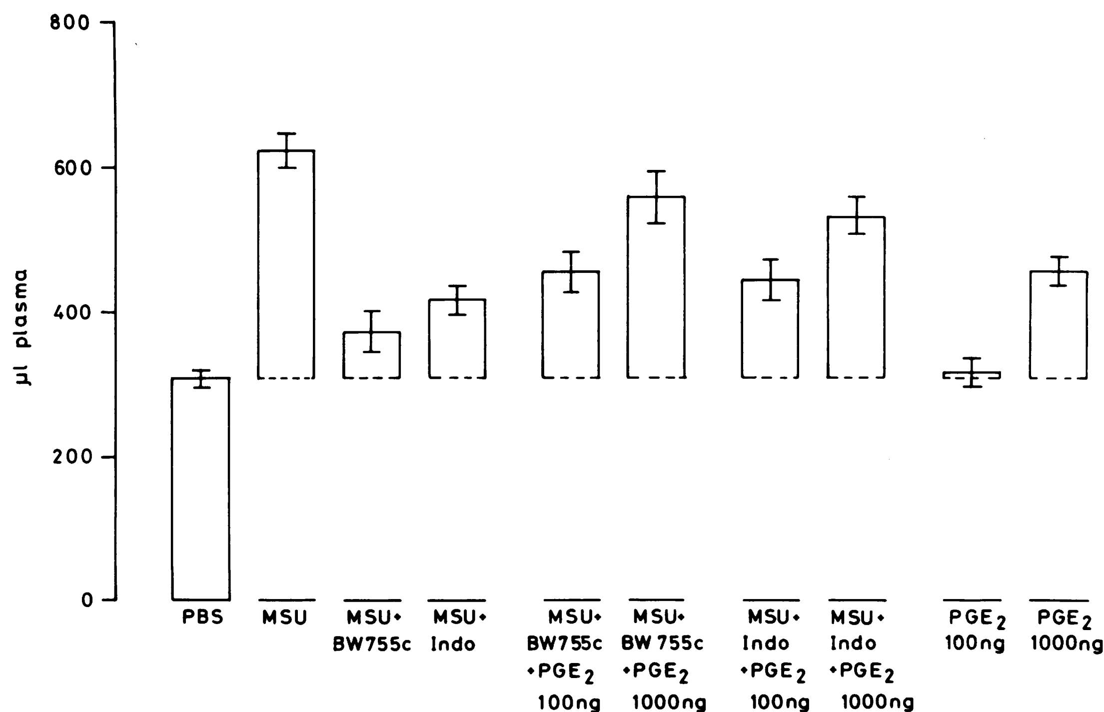

Fig. 2 *Plasma protein leakage induced by monosodium urate (MSU) crystals (n=28) was measured by the accumulation of intravenously injected Evans blue dye in the air pouch. The effects of BW 755c (50 mg/kg; n=16) and indomethacin (2 mg/kg; n=13) on plasma leakage were determined, together with the ability of prostaglandin E_2 (PGE_2) to reverse the effects of BW 755c (n=14 and n=13 for doses of 100 ng/ml and 1000 ng/ml respectively) or indomethacin (n=11 and n=10 for doses of 100 ng/ml and 1000 ng/ml respectively). Prostaglandin E_2 alone was also injected into the air pouch at 100 ng/ml (n=12) or 1000 ng/ml (n=13). The dashed horizontal line represents the mean plasma leakage in response to phosphate buffered saline (PBS, n=22). Each bar represents the mean value and vertical lines represent the SEM.*

Discussion

We have confirmed our previous findings that the injection of MSU crystals into the rat subcutaneous air pouch initiates an acute inflammatory response with increased PMNL accumulation, plasma leakage, and generation of LTB_4.[10] These data contrast with those of Carlson *et al*, who did not find increased levels of LTB_4 in MSU induced synovitis in the dog,[14] but are in agreement with the observation of raised LTB_4 levels in gouty synovial fluid[1] and in PMNL supernatants incubated with MSU crystals.[2]

LTB_4 increases PMNL accumulation in vivo in a number of species, including man,[4 15 16] rabbits,[5 17] and guinea pigs.[3] We did not observe an increase in PMNL accumulation or plasma leakage in the air pouch in response to LTB_4. A similar lack of efficacy of LTB_4 in producing PMNL accumulation has been reported by Foster *et al* in the rat[18] and by Carlson *et al* in the dog.[14] These data suggest clear species differences in the in vivo chemotactic response to LTB_4. Similarly, Kreisle *et al* found that LTB_4 was not chemotactic for rat PMNLs in vitro,[19] though human PMNLs were responsive, and the rat PMNLs were responsive to other stimuli, such as zymosan activated plasma and formyl-Met-Leu-Phe. We have recently confirmed the absence of a chemotactic response to LTB_4 by rat PMNLs in vitro (O'Callaghan *et al*, unpublished observation). The absence of an in vivo chemotactic response to LTB_4 in rats provides an explanation for the inability of BW 755c to inhibit MSU induced PMNL accumulation. Thus LTB_4 does not appear to be the mediator of MSU induced PMNL accumulation in the air pouch, though the identity of the mediator is currently unknown.

Both BW 755c and indomethacin inhibited MSU induced plasma leakage, suggesting a role for prostaglandins in this response. The ability of exogenous prostaglandin E_2 to overcome the inhibitory effect of either BW 755c or indomethacin further supports prostaglandin E_2 as a mediator of increased plasma leakage in this model. It must be noted, however, that prostaglandin E_2 only showed this effect at a concentration of 1000 ng/ml and was ineffective at 100 ng/ml. This contrasts with a concentration of only 12·4 ng/ml of prostaglandin E_2 in the MSU induced exudate. Therefore, the interpretation of a role for PGE_2 in mediating MSU induced plasma leakage must be treated with some caution.

These data raise two important questions. Firstly, what is the biological role of LTB_4 generated in response to MSU crystals in the rat air pouch. The data presented here clearly indicate that LTB_4 does not mediate either PMNL accumulation or increased plasma leakage, but these are only two of the many biological properties ascribed to LTB_4. It is well documented that LTB_4 stimulates a number of PMNL functions, including the release of lysosomal enzymes and increased cell adhesiveness.[20] These properties of LTB_4 may potentially be of importance in the removal and clearance of MSU crystals.

The second question which needs addressing is what is the identity of the mediator(s) of MSU induced PMNL accumulation in this model. Our current investigations suggest the presence of a heat stable compound of molecular weight greater than 5000. A number of possibilities exist, including the complement derived peptide C5a, interleukin 1, or the crystal induced chemotactic factor described by Spilberg and Mandell.[21] Although urate crystals can activate complement,[22] we believe that C5a is unlikely to be the mediator of PMNL accumulation in this model as reduction of plasma leakage by either BW 755c or indomethacin was not accompanied by a reduction in cell accumulation. Because C5a is derived from C5, present in plasma, one might expect that a reduction in plasma proteins would make less C5a available and consequently cause a reduction in the signal for PMNL accumulation. Interleukin 1 has recently been produced from macrophages cultured in the presence of urate crystals.[23] Because the lining of the rat air pouch contains macrophage-like cells[24] it is possible that interleukin 1 could be the mediator of PMNL accumulation seen in this model.

The answers to these questions should shed interesting light on the nature of the inflammatory response to MSU crystals in the air pouch and may provide important insights to the pathogenesis of gout in man.

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