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Blockade of leukotriene B_4 prevents articular incapacitation in rat zymosan-induced arthritis

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Abstract

We investigated whether leukotrienes mediate cell influx and articular incapacitation in zymosan-induced arthritis. Rats received 1 mg zymosan intra-articularly (i.a.). The hyperalgesia was measured using the rat articular incapacitation test. Cell influx, leukotriene B_4 and prostaglandin E_2 levels were assessed in the joint exudate, at 6 h. Groups received either the leukotriene B_4 synthesis inhibitor MK 886 (3-[1-(4-chlorobenzyl)-3-*t*-butyl-thio-5-isopropylindol-2-yl)]-2,2-dimethylpropanoic acid 30 min before or 2 h after the zymosan; 0.3–3 mg kg^{-1} i.p.), the leukotrienes synthesis inhibitor BWA_4C (*N*-(3-phenoxycinnamyl)-acetohydroxamic acid—2 h after the zymosan; 10 μg i.a.) or the peptido-leukotrienes antagonist sodium montelukast (30 min before and 2 h after the zymosan; 10 mg kg^{-1} per os). MK 886 inhibited the articular incapacitation and cell influx, while reducing leukotriene B_4, but not prostaglandin E_2 levels. BWA_4C inhibited the articular incapacitation. Sodium montelukast did not affect either of the parameters. The data suggest that leukotriene B_4 is involved in cell influx and articular incapacitation in zymosan arthritis.

Keywords: Leukotrienes; Arthritis; Zymosan; Neutrophils; Hyperalgesia; Pain

1. Introduction

Limitation of movement secondary to joint hyperalgesia is a serious burden to patients presenting with inflammatory arthropathies. The rat knee-joint incapacitation test was designed to study articular incapacitation, defined as the inability of a rat with experimentally induced arthritis to walk normally (Tonussi and Ferreira, 1992). In the present study, we assumed that articular incapacitation reflects joint hyperalgesia following an inflammatory insult to the joint. Zymosan, a polysaccharide from yeast cell walls, produces a severe and erosive synovitis (Keystone et al., 1989) associated with acute increases in vascular permeability and cell migration followed by a progressive synovitis (Gegout et al., 1994). We have used the zymosan-induced arthritis model as a tool to investigate the effect of inflammatory mediators in the synovial oedema, cell influx and also in the inflammatory hyperalgesia that happens in this model in the rat (Rocha et al., 1999).

The participation of neutrophils in the pathogenesis of tissue lesions in arthritis has long been recognized. These cells are predominant in the synovial exudates of a variety of inflammatory arthropathies, including gout, Reiter's disease

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and rheumatoid arthritis (Harris, 1990). With respect to rheumatoid arthritis, although neutrophils do not seem to be responsible for the perpetuation of the chronic synovitis, these cells are important sources of substances that promote cartilage breakdown as well as bone resorption, such as reactive oxygen and nitrogen species, lysosomal enzymes and metalloproteases (Hampton et al., 1998). Indeed, the efficacy of the so-called disease modifying anti-rheumatic drugs in rheumatoid arthritis is usually associated with their ability to decrease neutrophil influx into the inflamed joints (Kraan et al., 2000; Tak and Bresnihan, 2000). Therefore, strategies to limit neutrophil trafficking and/or activation have received attention as potential alternatives to treat arthritis.

Amongst the substances involved in neutrophil recruitment to inflammatory foci, leukotrienes appear to be very relevant. Leukotriene B_4 is a very potent chemotactic agent for neutrophils both in vitro and in vivo (Crooks and Stockley, 1998). The nonsteroidal anti-inflammatory drugs currently used in clinical practice act through inhibiting the cyclooxygenases, thereby limiting prostanoids production, without blocking lipoxygenases and the subsequent leukotrienes production. Therefore, it was postulated that a dual inhibitor could be more efficacious as an anti-inflammatory compound (Unangst et al., 1994). The pharmacological strategies to block leukotrienes include either 5-lipoxygenase inhibition, which is a rate-limiting step in leukotrienes synthesis or the use of selective leukotrienes receptor antagonists. The compounds BWA_4C and MK 886 block the production of all leukotrienes, the latter through binding to the 5-lipoxygenase activating peptide (FLAP) (Depre et al., 1993; Tateson et al., 1988). On the other hand, the compound sodium montelukast, which is currently used as a treatment for asthma, selectively binds to cysteinyl receptors, functioning as a receptor antagonist for cysteinyl leukotrienes (Vianna and Martin, 1998).

Though leukotrienes, mainly leukotriene B_4, are considered to be important mediators of neutrophil migration, they have also been linked to pain mechanisms, apparently as a secondary phenomena resulting from their activity as neutrophil chemoattractants (Levine et al., 1984). In the present study, we investigated the effect of MK 886, BWA_4C and sodium montelukast in the zymosan-induced arthritis in rats, focusing on the possible correlation between the hyperalgesic effect of leukotrienes and cell migration.

2. Materials and methods

2.1. Induction of the zymosan arthritis

Male Wistar rats (180–220 g) from our own animal facilities were used throughout the experiments. All experiments were designed to minimize animal suffering and to use the minimum number of animals associated with valid statistical evaluation. Surgical procedures and animal treatments were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (DHEW Publication, Bethesda, MD, USA).

2.2. Evaluation of articular incapacitation

After light ether anesthesia, rats were subjected to the intra-articular (i.a.) injection of 1mg zymosan (50 μl total volume), dissolved in sterile saline, into their right knee joints. Control animals received saline. We used the rat knee-joint incapacitation test, as described previously, as a measure of the inflammatory joint hyperalgesia (Tonussi and Ferreira, 1992). Briefly, after zymosan injection, animals were put to walk on a steel rotary drum (30 cm wide×50 cm diameter), covered with a fine-mesh non-oxidizable wire screen, which rotates at 3 rpm. Specially designed metal gaiters were wrapped around both hind paws. After placement of the gaiters, the animals were allowed to walk for habituation. The right paw was then connected via a simple circuit to a microcomputer data input/output port. The paw elevation time is the time the animal walks failing to touch the cylinder with the injected hind paw, during a 60-s period, which is directly proportional to the articular incapacitation. The paw elevation time was measured at baseline and then hourly, until sacrifice, at 6 h after injection of the zymosan, irrespective of the drug treatments. Animals that received the compounds prior to or 2 h after the zymosan were regarded as pertaining to the prophylactic and therapeutic groups, respectively.

2.3. Evaluation of cell influx, leukotriene B_4 and prostaglandin E_2 release

Six hours after injection of the zymosan, the animals were anaesthetized (chloral hydrate 400 mg kg^{-1} i.p.), killed by cervical dislocation and ex-sanguinated. The synovial cavity of the knee joints was then washed with 0.4 ml saline containing 5 U/ml heparin. The synovial exudates were collected by aspiration. Total and differential cell counts were then performed using a Neubauer chamber and Diff-Quick™ staining, respectively. After centrifugation (500 g/10 min), the supernatant was stored at −70 °C and used for determination of leukotriene B_4 and prostaglandin E_2 concentrations using commercially available enzyme-linked immunosorbent assay (ELISA) kits.

2.4. Drug treatments

In order to analyze the prophylactic and the therapeutic effect of the compounds, MK 886 (0.3 or 3 mg kg^{-1} i.p.) was injected 30 min prior to or 2 h after injection of zymosan, respectively; sodium montelukast (10 mg kg^{-1} per os) was administered 30 min before and 2 h after injection of zymosan. Non-treated groups consisted of rats that received just 1 mg zymosan i.a., followed by 0.9% w/v sterile NaCl i.p. A group that received 10% carboximethylcellulose in saline, per os, was used as a vehicle control for

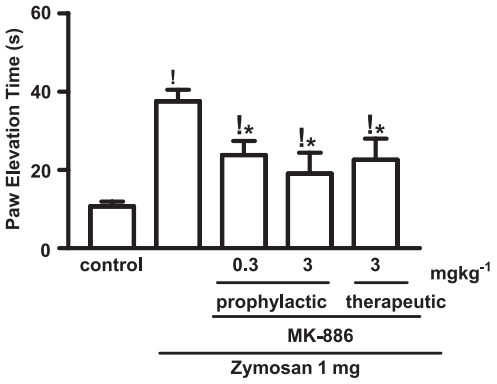

Fig. 1. Effect of the prophylactic or therapeutic administration of MK 886 on articular incapacitation in zymosan arthritis. The articular incapacitation was measured hourly, as the increase in the paw elevation time, over 6 h after injection of 1mg zymosan (i.a.). MK 886 (0.3 or 3 mg kg^{-1} i.p.) was injected 30 min prior to or 2 h after the zymosan. All groups but the control animals received zymosan i.a. The non-treated group was given saline i.p. followed by the zymosan. MK 886 significantly ($P<0.05$) reduced the articular incapacitation regardless of the injection strategy. Results are expressed as the mean±S.E.M. of maximal paw elevation time; $n=6$ animals for each group. $^{!}P<0.05$ compared to control; $^{*}P<0.05$ compared to zymosan.

the orally administered compounds. In order to study the effect of the local administration of a lipoxygenase inhibitor, a group received the compound BWA_4C (10 μg in 50 μl of saline, i.a.) dissolved in 2% dimethylsulfoxide (DMSO) either 30 min before or 2 h after injection of the zymosan. The control for this group received 50 μl of a 2% DMSO solution i.a. 30 min before or 2 h after the zymosan. There were six animals for each group.

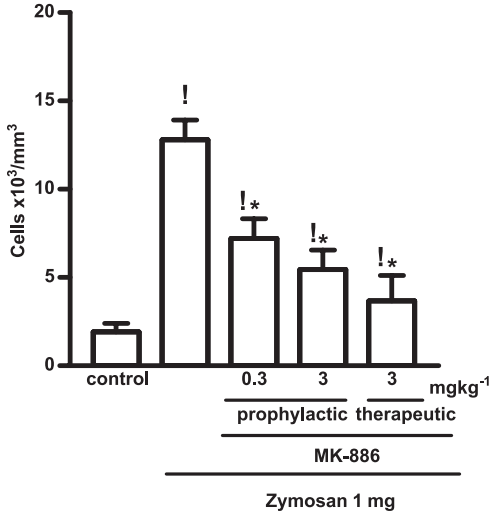

Fig. 2. Effect of the prophylactic or therapeutic administration of MK 886 on the cell influx into the joints measured at 6 h after injection of 1 mg zymosan (i.a.). MK 886 (0.3 or 3 mg kg^{-1} i.p.) was injected 30 min prior to or 2 h after the zymosan. All groups but the control animals received zymosan. The control group was given saline (i.a.). The non-treated group was given saline i.p. followed by the zymosan. MK 886 significantly ($P<0.05$) reduced the cell influx regardless of the injection strategy. Results are expressed as the mean±S.E.M. of total leukocytes at 6 h of zymosan arthritis; $n=6$ animals for each group. $^{!}P<0.05$ compared to control; $^{*}P<0.05$ compared to zymosan i.a.

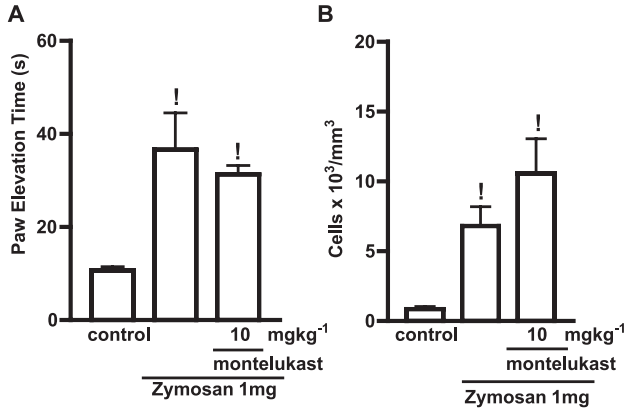

Fig. 3. (A) Effect of the administration of sodium montelukast on the articular incapacitation in zymosan arthritis. Sodium montelukast (10 mg kg^{-1} per os) was injected 30 min prior to and 2 h after injection of 1 mg zymosan (i.a.). The articular incapacitation was measured hourly, as the increase in the paw elevation time, over 6 h after injection of 1 mg zymosan (i.a.). All groups but the control animals received zymosan. The control group was given saline (i.a.). The non-treated group was given saline i.p. followed by the zymosan. Results are expressed as the mean±S.E.M. of maximal paw elevation time; $n=6$ animals for each group. $^{!}P<0.05$ compared to control. (B) Effect of sodium montelukast on the cell influx into the joints measured at 6 h after injection of 1 mg zymosan (i.a.). Sodium montelukast (10 mg kg^{-1} per os) was injected 30 min prior to and 2 h after the zymosan. Results are expressed as the mean±S.E.M. of total leukocytes; $n=6$ animals for each group. $^{!}P<0.05$ compared to control.

2.5. Drugs and reagents

Zymosan and MK 886 were purchased from Sigma, St. Louis, USA; BWA_4C was a kind donation of Wellcome,

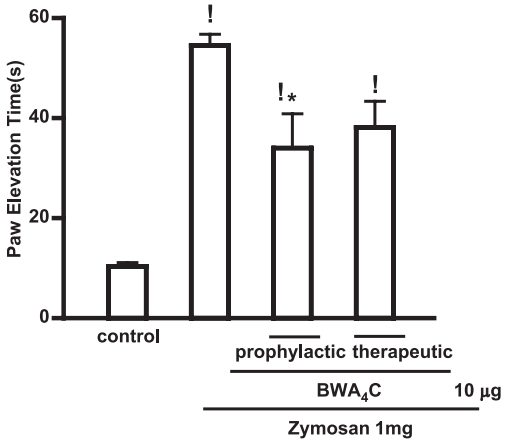

Fig. 4. Effect of the prophylactic or therapeutic administration of BWA_4C on the articular incapacitation in zymosan arthritis. The articular incapacitation was measured hourly, as the increase in the paw elevation time, over 6 h after injection of 1 mg zymosan (i.a.). BWA_4C (10 μg i.a.) was injected 30 min prior to or 2 h after the zymosan. All groups but the control animals received zymosan. The control group was given saline (i.a.). The non-treated group was given saline i.a. 30 min before the zymosan. BWA_4C significantly ($P<0.05$) reduced the articular incapacitation when given prophylactically. Results are expressed as the mean±S.E.M. of maximal paw elevation time; $n=6$ animals for each group. $^{!}P<0.05$ compared to control; $^{*}P<0.05$ compared to zymosan.

London, UK. Sodium montelukast was purchased from Merck Sharp Dohme, São Paulo, Brazil. The leukotriene B_4 and prostaglandin E_2 ELISA kits were purchased from Cayman Chem., USA.

2.6. Statistics

Results are expressed as mean±standard error of the mean (S.E.M.). Statistically significant differences between groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test. $P<0.05$ was considered significant.

3. Results

3.1. Effect of systemic leukotriene blockade

The effect of the administration of MK 886 and sodium montelukast on the articular incapacitation of rats subjected to the zymosan arthritis is shown in Figs. 1 and 3, respectively. MK 886 significantly ($P<0.05$) inhibited the articular incapacitation both when given prophylactically and therapeutically. Sodium montelukast had no effect on the articular incapacitation.

The effect of the prophylactic or therapeutic administration of MK 886 or sodium montelukast on the cell influx into the joint exudates of rats subjected to the zymosan arthritis is shown in Figs. 2 and 3, respectively. Whereas MK 886 significantly inhibited the cell influx, sodium montelukast did not modify the cell influx.

3.2. Effect of local leukotrienes synthesis blockade

Fig. 4 illustrates the effect of the administration of BWA_4C i.a. showing that this compound provoked a significant reduction of the articular incapacitation when injected prophylactically ($P<0.05$). Though there was a 30% reduction of the articular incapacitation when BWA_4C was injected therapeutically, it did not reach statistical significance.

Table 1
Effect of MK 886 on leukotriene B_4 and prostaglandin E_2 levels released into the joint exudates in zymosan-induced arthritis

	Leukotriene B_4 (pg/ml)	Prostaglandin E_2 (ng/ml)
Zymosan	200±14.7	1656±43
Zymosan+ MK 886	158±7.75*	1758±21

Rats were given 1 mg zymosan (i.a.). Leukotriene B_4 and prostaglandin E_2 levels released into the joint exudates at 6 h of arthritis were measured using ELISA. MK 886 (3 mg kg^{-1} i.p.) was given 30 min prior to the zymosan. Data represent mean±S.E.M.; $n=6$ animals for each group.

* $P<0.05$ compared to zymosan.

3.3. Effect of MK 886 on leukotriene levels

Table 1 shows that the administration of MK 886 significantly reduced the levels of leukotriene B_4, but not of prostaglandin E_2, obtained in the synovial exudates of rats, 6 h after the injection of zymosan, as compared to the non-treated group ($P>0.05$).

4. Discussion

The present study demonstrates, for the first time, that both local and systemic blockade of leukotrienes synthesis, but not the antagonism of cysteinyl leukotrienes receptors, inhibit articular incapacitation in an experimental arthritis model. Articular incapacitation, as measured in the present study, was shown to be inhibitable by classic analgesic and anti-inflammatory compounds using carrageenin as the inciting agent (Tonussi and Ferreira, 1992). Though oedema could be responsible for the articular incapacitation, previous data argue against this premise. Actually, intra-articular injection of dextran, despite eliciting joint oedema, does not lead to articular incapacitation (Tonussi and Ferreira, 1992). Moreover, the administration of nitric oxide synthase inhibitors prior to zymosan inhibits the articular incapacitation, while not altering joint oedema (Rocha et al., 2002). Apparently, local release of inflammatory mediators and cell infiltration would account for the articular incapacitation. In the present study, we used this parameter as a measure of hyperalgesia happening in the inflamed joint.

We observed that the leukotrienes synthesis inhibitor MK 886 reduced the hyperalgesia in zymosan-induced arthritis regardless of being administered prophylactically or therapeutically. The fact that MK 886 significantly reduced leukotriene B_4 levels in the joint exudates, while not altering prostaglandin E_2 release, point to a specific pharmacological activity of MK 886 as a 5-lipoxygenase inhibitor in this model. Using an immunecomplex arthritis model in rats, we have shown that leukotriene B_4 is released as early as 5 min after induction of arthritis (Rocha et al., 1997). In the zymosan-induced arthritis model in rats, we have obtained a significant release of leukotriene B_4 as early as 1 h after injection of the zymosan (our unpublished data). Since the cell influx into the joints in zymosan-induced arthritis starts at least 2 h after injection of the zymosan, it is clear that the production of leukotriene B_4 precedes the influx of the cells. Although neutrophils may be involved in the production of leukotrienes in this model, other cells, such as the resident synoviocytes, might also account for the almost immediate release of leukotriene B_4 after injection of the zymosan. Moreover, as MK 886 could prevent the inflammatory hyperalgesia and neutrophil influx when given after the administration of zymosan, it is reasonable to suppose that there is a sustained production of leukotriene B_4 that is preventable by the therapeutic administration of MK 886.

Similarly to what was observed with MK886, the intra-articular injection of the leukotriene synthesis inhibitor BWA_4C also significantly reduced the hyperalgesia. However, this effect reached statistical significance only when the compound was given prophylactically. BWA_4C is an acetohydroxamic acid which activity probably derives from the direct inhibition of the 5-lipoxygenase enzyme that is a rate-limiting enzyme in the leukotriene synthesis (Tateson et al., 1988). After oral administration, BWA_4C was shown to be more potent in inhibiting leukotriene B_4 synthesis rather than neutrophil influx after the subplantar injection of carrageenin in rats (Higgs et al., 1988). The dose of BWA_4C (10 μg/joint) used in the present study was shown to be able to inhibit eosinophil chemotaxis in an antigen-induced pleuritis (e Silva et al., 1992). Taken together, these results strongly argue for a participation of leukotrienes, mainly leukotriene B_4, in the inflammatory hyperalgesia in zymosan-induced arthritis.

As alluded to above, it has been proposed that the hyperalgesic activity of leukotriene B_4 is linked to its ability to promote neutrophil trafficking (Levine et al., 1984). Based on the well-known chemotactic activity of leukotriene B_4, we evaluated the cell influx into the joints. Our data have shown that the compound MK 886 significantly inhibited the cell influx in zymosan arthritis both when injected prophylactically and therapeutically. In the immunecomplex arthritis model in rats, we have shown that MK 886 also inhibited neutrophil migration, an effect that was associated with decreased release of leukotriene B_4 into the joint exudates (Rocha et al., 1997). Previous data obtained by our group suggest that joint hyperalgesia and neutrophil influx may be independent phenomena in zymosan arthritis. In this model, neutrophil influx is maximal at 6 h of arthritis, whereas the articular incapacitation, assumed as reflecting joint hyperalgesia, peaks between 3 and 4 h after injection of the zymosan (Rocha et al., 1999). Moreover, in the immunecomplex arthritis model, there is no demonstrable articular incapacitation, despite the neutrophil influx being similar to what is observed in zymosan arthritis (Rocha et al., 1999). However, based on the inhibitory effects of MK 886 on both neutrophil influx and hyperalgesia, our present data suggest that the analgesic effect obtained after leukotriene synthesis blockade is associated with and may be secondary to a reduction in neutrophil trafficking into the joints. The fact that the compound MK 886 reversed the inflammatory hyperalgesia and the leukotriene B_4 release into the joint exudates, without altering prostaglandin E_2 levels, argues for a major role of leukotrienes in the pain development in this model.

A previous study has shown that the selective leukotriene B_4 receptor antagonist LY293111Na inhibited the oedema and the neutrophil influx as well as cartilage and bone destruction in an antigen-induced arthritis model in guinea pigs. However, in that same study, LY293111Na showed only a slight inhibitory effect in the paw oedema of the antigen-induced arthritis model in rats (Kuwabara et al., 2002). Our data showing that leukotriene B_4 is involved in the articular incapacitation and neutrophil influx, however, argue for an important role for this mediator in rat arthritis. The different compounds and stimuli used might explain this apparent discrepancy between the two studies.

The ability of cysteinyl leukotrienes to promote increase in vascular permeability and to contract the inferior airways in hypersensitivity reactions has prompted the use of these compounds as a treatment option in asthmatic patients. Zafirlukast, a selective cysteinyl receptor antagonist, was recently shown to inhibit the inflammatory nociception after injection of carrageenin into the rat paw, a peripherally mediated pain model (Jain et al., 2001). However, in that same study, zafirlukast did not inhibit the centrally mediated pain evoked in the tail-flick and the acetic acid writhings test in mice. To our knowledge, there are no studies on the antinociceptive effect of cysteinyl receptor antagonists using arthritis models. Our data have shown that sodium montelukast did not inhibit either the inflammatory hyperalgesia or the cell migration into the joint exudates in zymosan arthritis. Montelukast and zafirlukast are considered equipotent in antagonizing cysteinyl leukotrienes (Coreno et al., 2000; Ravasi et al., 2002). In the above-mentioned study, the administration of either 2.5 or 5.0 mg/kg of zafirlukast significantly inhibited the hyperalgesia after carrageenin injection into the rat paw (Jain et al., 2001). Thus, the possibility of insufficient dose of sodium montelukast to explain our present results seems unlikely. Overall, these results reinforce the idea that leukotriene B_4, rather than cystenyl leukotrienes, is the main product of 5-lipoxygenase responsible for cell influx and hyperalgesia in the model.

The importance of leukotriene B_4 in arthritis cannot be overemphasized. A very recent clinical study has shown that an orally active leukotriene B_4 antagonist provided almost complete inhibition of the expression of the adhesion molecule CD11B/CD18 in the peripheral leukocytes from patients with rheumatoid arthritis, thus prompting the need for clinical studies to evaluate the possible clinical benefit of such compounds to these patients (Alten et al., 2004).

In summary, this study presents evidence that leukotriene B_4 is involved in both the influx of polymorphonuclear neutrophils into the joints and in the development of inflammatory hyperalgesia in the zymosan-induced arthritis in rats. Taking into account the well-known analgesic effect of the cyclooxygenase inhibitors, the designing of compounds able to inhibit both cyclooxygenase and lipoxygenase metabolites, mainly leukotriene B_4, may provide better analgesia in inflammatory arthropathies.

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