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Anti-nociceptive effect of thalidomide on zymosan-induced experimental articular incapacitation

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Abstract

The anti-nociceptive effect of thalidomide on zymosan-induced articular knee joint incapacitation in rats was investigated. Thalidomide (5–45 mg/kg), given 30 min before but not 2 h after the intra-articular injection of zymosan, inhibited the nociceptive response in a dose-dependent manner. Furthermore, thalidomide pretreatment significantly reduced the concentration of tumor necrosis factor-alpha (TNF-α, −68.4%) in the exudate of zymosan-injected joints, but not those of interleukin-1β, interleukin-6, CINC-1 or interleukin-10. The expression of TNF-α, determined by immunohistochemical staining, in synovial tissues obtained from articular joints injected with zymosan was also inhibited by thalidomide pretreatment. The anti-nociceptive effect of thalidomide was not reversed by the co-administration of an opioid receptor antagonist, naloxone, suggesting that endogenous opioids do not mediate the anti-nociceptive effect of thalidomide in this model. In conclusion, the anti-nociceptive activity of thalidomide in zymosan-induced articular incapacitation is associated with the inhibition of TNF-α by resident synovial cells.

Keywords: Thalidomide; Tumor necrosis factor; Hyperalgesia; Arthritis; Zymosan

1. Introduction

Peripheral inflammation is characterized by an increase in the sensitivity of nerves endings which is clinically referred to as hyperalgesia in humans and hypernociception in experimental animals (Millan, 1999). This condition has long been associated with the production and release of cytokines at the inflammatory site, such as tumor necrosis factor-α (TNF-α), interleukin-1β, interleukin-6 and interleukin-8, which trigger the release of the final nociceptive mediators, including eicosanoids and sympathomimetic amines. These final nociceptive mediators directly sensitize the nociceptors (Nakamura and Ferreira, 1987; Ferreira and Nakamura, 1979). TNF-α is a cytokine released during the inflammatory process, which has a pivotal role in the onset of inflammatory hypernociception (Cunha et al., 1992). Many cell types, including macrophages (Matthews, 1981), keratinocytes (Corsini and Galli, 1998), fibroblasts (Fujisawa et al., 1997) and neutrophils (Djeu et al., 1990) produce TNF-α. Mast cells not only produce this cytokine but are also able to store it in their granules (Gordon and Galli, 1991). Schwann cells, which are present in peripheral nerves, also produce TNF-α after nerve injury (Wagner et al., 1998).

Concerning the involvement of TNF-α in inflammatory pain, our group and others have demonstrated that it has hypernociceptive property. Intraplantar injection of TNF-α in rats produces both mechanical (Cunha et al., 1992; Woolf et al., 1997) and thermal (Perkins and Kelly, 1994; Woolf et al., 1997) hyperalgesia. Furthermore, it is released early during acute inflammation and triggers the release of a cascade of pro-nociceptive cytokines, including interleukin-1 and interleukin-

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6, which stimulate cyclooxygenase expression with the consequent production of prostaglandins. Furthermore, TNF-α also stimulates the production of chemokines, such as interleukin-8 and neutrophil chemoattractant-1 (CINC-1), which in turn stimulate the release of sympathomimetic amines (Cunha et al., 1992; Woolf et al., 1997; Lorenzetti et al., 2002). Both prostaglandin and sympathomimetic amines directly sensitize the nociceptor, as mentioned above. Moreover, TNF-α also triggers persistent hypernociception in rats by a mechanism dependent on the release of interleukin-1β and CINC-1 (Sachs et al., 2002). There is evidence in the literature that also points to the participation of TNF-α in neuropathic pain (Schafers et al., 2003; Sommer et al., 1998; Wagner et al., 1998).

The hyperalgesic effect of TNF-α on articular diseases has also been demonstrated in experimental models and in clinical studies. Tonussi and Ferreira, using the rat knee joint incapacitation test, demonstrated that TNF-α mediates carrageenin-induced joint incapacitation (Tonussi and Ferreira, 1999). Feldmann et al. developed the concept that the inhibition of TNF-α activity suppresses downstream interleukin-1 and interleukin-6 production and chronic inflammation in rheumatoid arthritis patients (Brennan et al., 1989; Williams et al., 1992; Feldmann et al., 1996). Reinforcing this concept, it was shown recently that humanized monoclonal antibodies against TNF-α are effective in suppressing inflammation, joint tissue lesions and pain observed in rheumatoid arthritis (Elliott et al., 1994; Furst et al., 2003). Furthermore, these antibodies are also effective in inflammatory bowel diseases, in which TNF-α is also involved in pathogenesis (Present et al., 1999).

Thalidomide (α-*N*-phthalimodoglutarimide), which was developed initially as a nonbarbiturate sedative and used as an anti-emetic for morning sickness, was taken off the market in 1961 after it was linked to a spate of major birth defects. Thalidomide's resurgence came about from the unexpected discovery in 1964 of its surprising activity against a cutaneous complication of leprosy called type 2 reaction or erythema nodosum leprosum (Sheskin, 1965; Sampaio et al., 1993). Gradually, thalidomide was reintroduced for the treatment of other diseases, including severe mucosal ulcers (e.g., associated with HIV infection or Behçet's disease) (Youle et al., 1989; Hamuryudan et al., 1998), prurigo nodularis (Vandenbroek, 1980) and chronic graft-versus-host disease (Vogelsang et al., 1992) as well.

Recent reports describing that the main pharmacological effect of thalidomide is the reduction of tumor necrosis factor-alpha (TNF-α) production (Sampaio et al., 1991) have led to the suggestion that thalidomide may also be useful in the treatment of inflammatory conditions. This effect on TNF-α protein synthesis seems to be due to enhanced degradation of the mRNA of this cytokine (Moreira et al., 1993). Several open-label studies and case reports have described the beneficial effect of thalidomide in Crohn's disease (Ehrenpreis et al., 1999; Bariol et al., 2002), rheumatoid arthritis (Gutierrez-Rodriguez et al., 1989; Huizinga et al., 1996), ankylosing spondylarthritis (Wei et al., 2003), systemic sclerosis (Oliver et al., 2000), cutaneous lupus erythematosus (Knop et al., 1983; Stevens et al., 1997) and a few other systemic disorders.

We have previously demonstrated that thalidomide exerts an anti-nociceptive effect on experimental inflammatory mechanical hypernociception and in the mouse writhing model, and that this effect is due to the inhibition of the production of TNF-α by resident inflammatory cells (Ribeiro et al., 2000a). Taking these findings into account, in the present study, we investigated the possible anti-nociceptive effect of thalidomide in an experimental model of inflammatory articular nociception, the zymosan-induced knee joint incapacitation test in rats. The limitation of movement secondary to joint hyperalgesia is a serious burden to patients presenting with inflammatory arthropathies. We assume that the knee-joint incapacitation observed in the test used in the present study reflects the joint hyperalgesia following an inflammatory insult to the joint. It was observed that thalidomide inhibits the zymosan-induced articular incapacitation by a mechanism associated with the inhibition of TNF-α production by resident synovial cells.

2. Materials and methods

2.1. Animals

Male Wistar rats (180–200 g) from the animal colony of the Federal University of Ceará were used. All experiments were conducted in accordance with the European Community guidelines for the use of experimental animals and were approved by the Ethics Committee of the School of Medicine of Ribeirão Preto (University of São Paulo) and of the School of Medicine of the Federal University of Ceará.

2.2. Rat knee joint incapacitation test

The rat knee joint incapacitation test is described in detail elsewhere (Tonussi and Ferreira, 1992). In this test, a computer-assisted device measures the length of time that a specific hind paw fails to touch the surface of a rotating cylinder in a 1-min period (paw elevation time). In normal animals, paw elevation time is approximately 10–15 s. In our experiments, incapacitation was studied in animals injected with zymosan (1 mg/animal; Rocha et al., 1999) into the knee joints, and the period for which the hind paw failed to touch the rotating cylinder was interpreted as being proportional to the pain felt by the animal. Paw elevation time was measured before zymosan administration (control time, T_0) and, thereafter, every hour for 4 h (T_1, T_2, T_3 and T_4).

2.3. Detection of TNF-α, interleukin-1β, interleukin-10, interleukin-6 and CINC-1 present in articular cavity exudate of joints injected with zymosan

TNF-α, interleukin-1β, interleukin-10, interleukin-6 and CINC-1 concentrations were determined in rat knee joint exudates 2 h after intra-articular injection of saline (50 μl) or zymosan (1 mg/50 μl). The articular cavity was washed twice

with 200 μl of heparinized saline and the pooled washes then centrifuged. The supernatant was used to determine the concentrations of TNF-α interleukin-1β, interleukin-10, interleukin-6 and CINC-1 by enzyme-linked immunosorbent assay (ELISA), as described previously (Cunha et al., 1993). Briefly, microtiter plates were coated overnight at 4 °C with antibody against rat TNF-α, interleukin-1β, interleukin-1, interleukin-6 or CINC-1 (10 μg/ml). After blocking the plates, the samples and standard at various dilutions were added in duplicate and incubated at 4 °C for 24 h. The plates were washed three times with buffer, and a second biotinylated polyclonal antibody diluted 1/1000 was added (100 μl/well). After further incubation at room temperature for 1 h, the plates were washed and 100 μl of avidin–horseradish peroxidase diluted 1:5000 were added. The color reagent *o*-phenylenediamine (OPD; 100 μl) was added 15 min later, and the plates were incubated in the dark at 37 °C for 15–20 min. The enzyme reaction was stopped with H_2SO_4, and absorbance was measured at 490 nm. The results are reported as means ± S.E.M. for four animals.

2.4. Immunohistochemical reaction for tumor necrosis factor

Immunohistochemistry for TNF-α was performed on synovial tissue from rat knee joint using the streptavidin–biotin–peroxidase method (Hsu et al., 1981) in formalin-fixed, paraffin-embedded tissue sections (4 μm thick), mounted on poly-L-lysine-coated microscope slides. The sections were deparaffinized and rehydrated through xylene and graded alcohols. After antigen retrieval, endogenous peroxidase was blocked (15 min) with 3% (v/v) hydrogen peroxide and washed in phosphate-buffered saline (PBS). Sections were incubated overnight (4 °C) with primary rabbit anti-rat TNF-α antibody diluted 1:100 in PBS plus bovine serum albumin (PBS-BSA). The slides were then incubated with biotinylated goat anti-rabbit; diluted 1:400 in PBS-BSA. After washing, the slides were incubated with avidin–biotin–horseradish peroxidase conjugate (Strep ABC complex by Vectastain® ABC Reagent and peroxidase substrate solution) for 30 min, according to the Vectastain protocol. TNF-α was visualized with the chromogen 3,3′-diaminobenzidine (DAB). Negative control sections were processed simultaneously as described above but with the first antibody being replaced by PBS-BSA 5%. None of the negative controls showed TNF-α immunoreactivity. Slides were counterstained with Harry's hematoxylin, dehydrated in a graded alcohol series, cleared in xylene and coverslipped.

2.5. Experimental protocol

2.5.1. Evaluation of thalidomide in the rat knee joint incapacitation test induced by zymosan

Rats were pretreated with vehicle (saline plus dimethyl sulfoxide) or thalidomide (5–45 mg/kg; i.p.), and 30 min afterward, zymosan (1 mg/joint) was injected intra-articularly in a volume of 50 μl. Incapacitation was measured as described above.

2.5.2. Evaluation of post-treatment with thalidomide in the rat knee joint incapacitation test induced by zymosan

Rats were injected intra-articularly with zymosan (1 mg/joint) in a volume of 50 μl, and 1 h afterward, vehicle or thalidomide (45 mg/kg; i.p.) was administered. Incapacitation was measured as described above.

2.5.3. Evaluation of naloxone in anti-nociceptive effect of thalidomide on zymosan-induced knee joint incapacitation in rats

Rats were pretreated s.c. with saline or naloxone (2 mg/kg). Fifteen minutes later, they were injected i.p. with vehicle, thalidomide (45 mg/kg) or morphine (5 mg/kg). Thirty minutes afterward, zymosan (1 mg/joint) was injected intra-articularly and knee joint incapacitation was determined as described above.

2.5.4. Effect of intraperitoneal thalidomide pretreatment on TNF-α, interleukin-1β, interleukin-10, interleukin-6 and CINC-1 production in articular exudates from rat knee joints stimulated with zymosan

Rats were pretreated i.p. with vehicle or thalidomide (45 mg/kg) 30 min before the intra-articular administration of zymosan (1 mg/joint). The control group received only an intra-articular injection of saline. After 2 h, the joints were washed with PBS plus heparin, and the collected fluids were centrifuged. The concentrations of the cytokines in the supernatant were determined by ELISA as described above.

2.5.5. Effect of intraperitoneal thalidomide pretreatment on immunohistochemical detection of tumor necrosis factor in synovial tissue of rat knee joints stimulated with zymosan

Rats were pretreated i.p. with vehicle or thalidomide (45 mg/kg) and 30 min later, zymosan was injected intra-articularly into the right knee joint (1 mg/joint). The naive group received only intra-articular saline injection. After 2 h, areas of the synovial tissue of the injected joints were removed and the immunohistochemical protocol was applied to these samples as described above.

2.6. Drugs, cytokines and antibodies

The following drugs were used: thalidomide (FUNED, Fundação Ezequiel Dias, Belo Horizonte, Brazil), zymosan A (Sigma-Aldrich, St Louis, MO), goat anti-rat TNF-α primary antibody and biotinylated anti-goat IgG antibody (NIBSC; National Institute for Biological Standards and Control, UK). Biotinylated anti-goat IgG antibody used in immunohistochemistry, Vectastatin ABC detection system and VIP substrate kit were from Vector Laboratories, Burlingame, CA.

Zymosan was diluted in a saline, and thalidomide was diluted in 0.4% dimethyl sulfoxide in saline (vehicle).

2.7. Data analysis

Results are presented as means ± S.E.M. of measurements made on at least four to six animals in each group. Differences

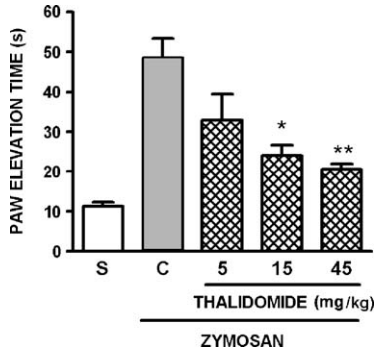

Fig. 1. Effect of pretreatment with thalidomide on zymosan-evoked articular incapacitation in rats. Thalidomide (5–45 mg/kg) was injected i.p., and 30 min later, zymosan (1 mg/joint; 50 μl) was injected intra-articularly into the right knee joint. Paw elevation time was measured before and after zymosan administration over a 1-min period until the fourth hour. Animals receiving vehicle i.p. injection before zymosan were designated the control group (C) and S indicate the group that received saline intra-articular instead of zymosan. The bars show means of maximal values obtained between the third and fourth hour after zymosan injection. Results are expressed as means±S.E.M. of the paw elevation time (s) for groups of six rats. Asterisks indicate statistically significant differences between groups and C group (*$P<0.05$ and **$P<0.01$; Turkey ANOVA).

between responses were evaluated by analysis of variance (ANOVA) followed by Tukey's test. Statistical differences were considered to be significant at $P<0.05$.

3. Results

3.1. Effect of the pretreatment with thalidomide on rat knee joint incapacitation induced by zymosan

The intra-articular injection of zymosan (1 mg/joint) induces articular incapacitation (Rocha et al., 1999; Vale et al., 2003).

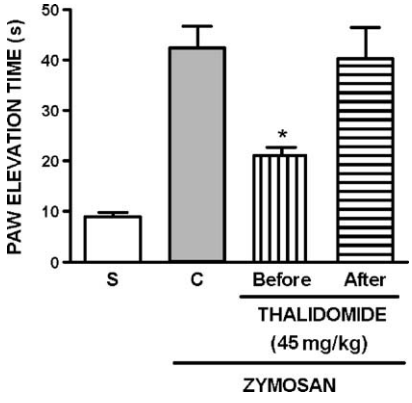

Fig. 2. Effect of post-treatment with thalidomide on zymosan-evoked articular incapacitation in rats. Thalidomide (45 mg/kg) was administrated 30 min before or 1 h after the intra-articular zymosan (1 mg/joint; 50 μl) injection. The control group (C) was administrated with vehicle 30 min before zymosan injection. The group indicated as S received an intra-articular injection of saline instead of zymosan. Paw elevation time was measured before and after zymosan administration over a 1-min period until the fourth hour. The bars show means of maximal values obtained between the third and fourth hour after zymosan injection. Results are expressed as means±S.E.M. of the paw elevation time (s) for groups of six rats. Asterisks indicate statistically significant differences between groups and C group (*$P<0.05$; Turkey ANOVA).

Fig. 1 shows articular incapacitation determined between the third and fourth hour after zymosan injection, which is the interval of maximal incapacitation response (Rocha et al., 1999; Vale et al., 2003). Thalidomide at doses of 15 and 45 mg/kg, but not at a dose of 5 mg/kg, significantly inhibited zymosan-evoked articular hyperalgesia up to 59% ($P<0.01$).

However, when administered 1 h after zymosan, thalidomide did not inhibit articular incapacitation (Fig. 2).

3.2. Effect of naloxone on the anti-nociceptive activity of thalidomide in zymosan-induced knee joint incapacitation in rats

Pretreatment with naloxone at 2 mg/kg, 15 min before thalidomide (45 mg/kg), did not affect the anti-hypernociceptive activity of this drug in zymosan-induced articular incapacitation in rats. However, at this dose, naloxone blocked the anti-nociceptive activity of morphine (5 mg/kg) in zymosan-induced articular incapacitation (Fig. 3). The injection of naloxone (2 mg/kg) in naive animals did not modify the nociceptive response (data not shown).

3.3. Effect of thalidomide pretreatment on TNF-α, interleukin-1β, interleukin-10, interleukin-6 and CINC-1 production in articular exudates from rat knee joints stimulated with zymosan

The exudates harvested from rat articular joints stimulated with zymosan (1 mg/joint) showed significant amounts of

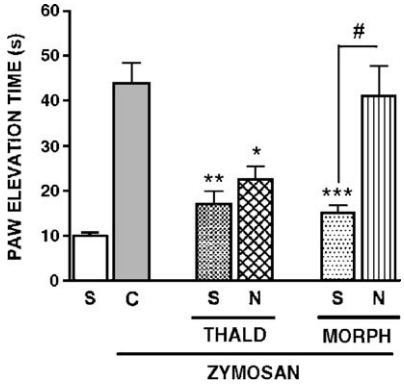

Fig. 3. Effect of pretreatment with naloxone on the antinociceptive action of thalidomide or morphine in zymosan-induced knee joint incapacitation in rats. Rats were pretreated s.c. with vehicle (S) or naloxone (N; 2 mg/kg). Fifteen minutes later, they were pretreated with thalidomide (THALD; 45 mg/kg) or morphine (MORPH; 5 mg/kg) i.p., and 30 min afterward, zymosan (1 mg/joint, 50 μl) was injected intra-articularly. The paw elevation times were measured before and after zymosan administration, over a 1-min period until the fourth hour. The bars show means of maximal values obtained between the third and fourth hour after zymosan injection ±S.E.M. for groups of six rats. Asterisk indicates a statistically significant difference (*$P<0.05$, **$P<0.01$; ***$P<0.001$; Turkey ANOVA) comparing the group injected with zymosan and pretreated with thalidomide plus vehicle or with morphine plus vehicle with the non-treated group (C; group injected with zymosan and pretreated only with saline). $^{\#}$Indicates a statistically significant difference comparing the group injected with zymosan and pretreated with morphine plus naloxone with the group injected with zymosan and pretreated with morphine plus vehicle ($^{\#}P<0.001$; Turkey ANOVA).

TNF-α, interleukin-1β, interleukin-6 and CINC-1 compared with the fluid harvest from articular joints injected with saline. Interleukin-10 was not increased by intra-articular zymosan injection, when compared to basal levels. The pretreatment of the animals with thalidomide (45 mg/kg; i.p.) caused a significant decrease of TNF-α release (−68.4%), but not of interleukin-1β, interleukin-6 or CINC-1 when compared with the zymosan-treated group pretreated with vehicle (Fig. 4).

3.4. Effect of thalidomide pretreatment on immunohistochemical detection of tumor necrosis factor in synovial tissue of rat knee joints stimulated with zymosan

Immunohistochemical staining of TNF-α increased in synovial cells of rats pretreated with zymosan (Fig. 5, panel B) as compared to the basal immunostaining present in the synovial tissue of naive rats (Fig. 5 panel C). Immunostaining of TNF-α in the synovial cells was clearly decreased by the pretreatment of the animals with thalidomide (Fig. 5, panel D). No immunostaining of TNF-α was found in the negative control, that is, synovial tissue incubated in the absence of anti-rat-TNF-α antibody (Fig. 5, panel A). Additionally, in animals treated with zymosan, we found synoviocyte hyperplasia, adipose synovium destruction and inflammatory cell infiltration (Fig. 5, panel B) compared to the naive group (Fig. 5, panel C). Synovium hyperplasia and adipose synovium destruction were prevented by thalidomide (Fig. 5, panel D).

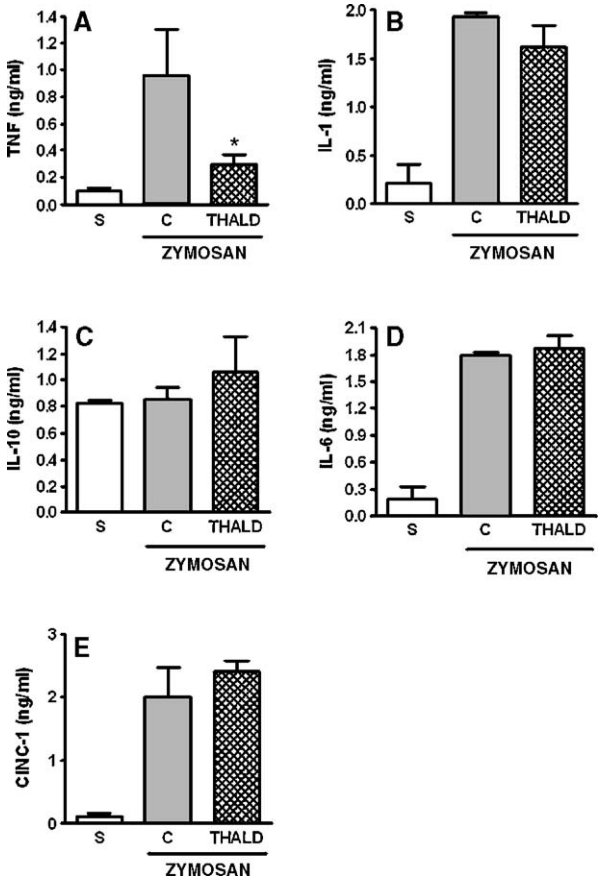

Fig. 4. Effect of thalidomide pretreatment on TNF-α, interleukin-1β, interleukin-10, interleukin-6 and CINC-1 production in knee joints injected with zymosan. The animals were injected intra-articularly with saline (50 μl joint^{-1}) or zymosan (1 mg/joint; 50 μl), and 2 h later, the articular cavities were washed and the concentrations of TNF-α (panel A), interleukin-1β (IL-1-β, panel B), interleukin-10 (IL-1, panel C), interleukin-6 (IL-6, panel D) and CINC-1 (panel E) in the exudates were determined by ELISA. Thalidomide (THALD; 45 mg/kg) or vehicle (C) was given i.p. 30 min before intra-articular zymosan injection. The groups indicated as S received an intra-articular injection of saline instead of zymosan. Results are reported as means ± S.E.M. of four wells and are representative of two different experiments. Asterisk indicates a statistically significant difference between groups and respective controls (*$P<0.05$; Turkey ANOVA).

4. Discussion

We have previously shown the anti-nociceptive effect of thalidomide in two experimental pain models: mechanical plantar hypernociception and the writhing test. The anti-nociceptive effect correlated with the inhibition TNF-α production (Ribeiro et al., 2000b). It is in accordance with the well-accepted pharmacological effect of thalidomide, that is, the prevention of TNF-α production by enhancing its mRNA degradation (Moreira et al., 1993). Reinforcing the notion that the analgesic effect of thalidomide is a consequence of the inhibition of TNF-α production, studies from our group and others have shown that TNF-α play a crucial role in the onset of inflammatory nociception in different experimental animals and models (Cunha et al., 1991, 1992; Tonussi and Ferreira, 1999; Sachs et al., 2002; Junger and Sorkin, 2000; Opree and Kress, 2000; Sommer et al., 1998; Ribeiro et al., 2000b; Vale et al., 2004). Furthermore, other drugs, including pentoxifylline and etanercept, which inhibit production of TNF-α or of its receptors, show anti-inflammatory and analgesic properties in several inflammatory diseases (Anaya and Espinoza, 1995; Vale et al., 2004; Culy and Keating, 2002). In the present study, we demonstrated that thalidomide also has anti-nociceptive activity against zymosan-induced knee joint incapacitation in rats.

It seems that the incapacitation observed in this test reflects the joint hyperalgesia that follows an inflammatory insult to the joint (Rocha et al., 1999; da Rocha et al., 2004) or that is observed in patients presenting with inflammatory arthropathies (Schaible et al., 2002). The participation of TNF-α in rheumatoid arthritis and other inflammatory diseases can be illustrated by the detection of increased levels of this cytokine in the joints of rheumatoid arthritis patients, as well as in the target tissues of inflammatory diseases (Neale et al., 1989; Harris, 1990). Moreover, the administration of TNF-α blockers to these patients resulted in a great amelioration of signs and symptoms of the disease and, at least for rheumatoid arthritis, led to the reduction of structural joint damage, evidenced by reduced radiographic lesions as well as diminished joint hyperalgesia (Culy and Keating, 2002; Furst et al., 1999; Breedveld et al., 2004). Moreover, TNF-α is a major player in experimental arthritis among several animal species and models. In several studies, TNF-α actions are associated with leukocyte recruitment, articular destruction and nociception (Williams et al., 1992; Idogawa et al., 1997). Confirming these findings, we

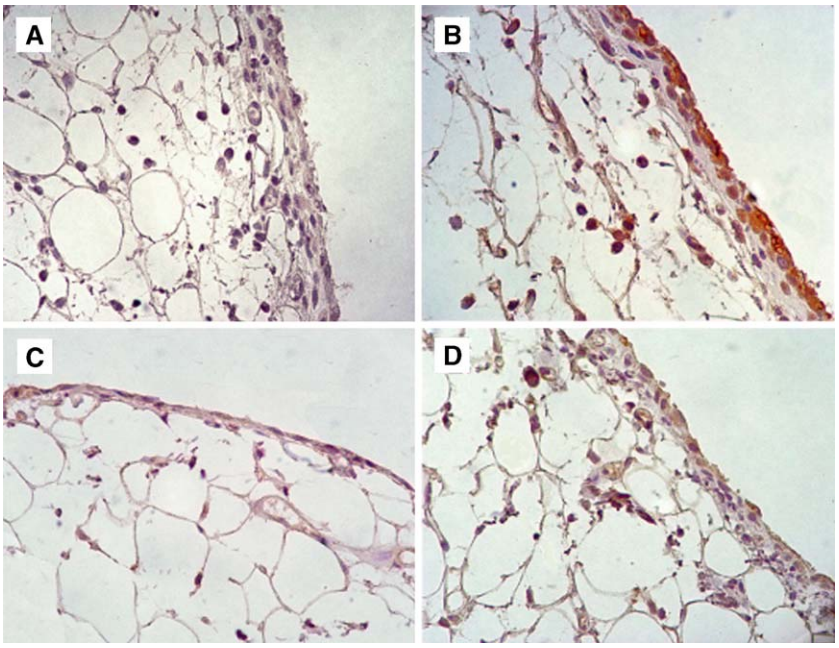

Fig. 5. Effect of thalidomide pretreatment on immunohistochemical detection of tumor necrosis factor in synovial tissue of rats knee joints stimulated with zymosan. The animals were injected intra-articularly with saline (50 μl joint^{-1}) or zymosan (1 mg/joint; 50 μl), and 2 h later, the synovial tissue was removed and processed for immunohistochemical staining. Panel A: negative control for immunostaining of TNF-α. A synovial tissue slice from a rat knee joint pretreated with i.p. vehicle and injected with zymosan was incubated in the absence of primary anti-rat TNF-α and no staining was obtained. Panel B: intense immunostaining for TNF-α in cells of synovial membrane of rats pretreated with i.p. vehicle and stimulated with intra-articular zymosan (1 mg/joint). Panel C: presence of understated immunostaining for TNF-α in synovial tissue of rats injected with intra-articular saline alone (naive animals). Panel D: reduction of immunostaining for TNF-α in cells of synovial tissue of rats pretreated with i.p. thalidomide (45 mg/kg) and stimulated with zymosan (1 mg/joint). DAB and Mayer's hematoxylin.

observed in the present study that there was a significant increase in TNF-α levels in joint exudates and also in the expression of TNF-α in the synovial tissue of zymosan-injected animals, and thalidomide treatment reduced both events. Thus, the use of thalidomide could be an alternative in controlling arthritic pain.

Post-treatment with thalidomide did not inhibit the hyperalgesic effect of zymosan, suggesting that after zymosan had induced the release of inflammatory mediators, thalidomide is not effective. In our previous study, we demonstrated that post-treatment with thalidomide is also ineffective in inhibiting carrageenin-induced plantar hypernociception (Ribeiro et al., 2000b). It is possible that in these investigated models, in which nociception is a short-lasting phenomenom, TNF-α is released mainly in the early hours after inflammatory insults, when a potentially effective concentration of thalidomide administered by post-treatment is not reached in the circulation. In fact, we demonstrated previously that a significant concentration of TNF-α is observed in the hind paw tissues injected with carrageenin or lipopolyssacharide, mainly in the first hour after the administration of stimuli (Cunha et al., 2000; Vale et al., 2004;). However, in chronic inflammatory pain, the thalidomide analgesic effect may occur even when it is administered in a post-treatment manner, because TNF-α is constantly being generated in this pathophysiological condition (Inglis et al., 2005).

Besides TNF-α, the levels of interleukin-1β, interleukin-6 and CINC-1 were also significantly increased in the joint exudates of zymosan-injected animals. The fact that thalidomide treatment reduced only TNF-α production confirms that it is a specific inhibitor of TNF-α production. There is evidence in the literature that the production of interleukin-1β, interleukin-6 and CINC-1 is increased in the joints of arthritic animals or patients and that they play an important role in the genesis of inflammatory pain (Cunha et al., 1992, 2000; De Jongh et al., 2003; Eastgate et al., 1988; Ferreira et al., 1988; Junger and Sorkin, 2000, Obreja et al., 2002). The observation that thalidomide did not affect the production of these pro-nociceptive cytokines explains, at least in part, the fact that thalidomide treatment did not block zymosan incapacitation completely. This finding also suggests that the production of interleukin-1β, interleukin-6 and CINC-1 in the joints of rats injected with zymosan did not depend on TNF-α production. This is in apparent contradiction to previous studies from our laboratory and others showing that TNF-α promotes nociception through the release of a cascade of cytokines (Cunha et al., 1992). For instance, in carrageenin-induced hypernocicpetion in the hind paw of rats, TNF-α is the first cytokine released and induces the production of interleukin-1β and CINC-1 (Cunha et al., 1992), which triggers the release of prostagladins and sympathomimetic amines, respectively. In accordance, thalidomide at similar doses in the present study blocked completely carrageenin-induced mechanical hypernociception (Ribeiro et al., 2000b). However, in mouse paw tissue, TNF-α stimulated the release of interleukin-1β but not of KC (related rat CINC-1 chemokine) (Cunha et al., 2005). The analgesic effect of thalidomide was not investigated in this model. Thus, the

mechanism by which TNF-α induces nociception depends of the species and model.

Thalidomide has been shown to stimulate the production of interleukin-10 in vivo (Moreira et al., 1997). We have also shown that interleukin-10 and also interleukin-4 and interleukin-13 inhibit inflammatory hypernociception in different species and models due to their capacity to inhibit the production of pro-nocicpetive cytokines and also the expression of COX-2 (Poole et al., 1995; Cunha et al., 1999; Lorenzetti et al., 2001; Vale et al., 2003). However, in zymosan-induced incapacitation, it seems that interleukin-10 is not involved in the analgesic effect of thalidomide, since we did not find enhancement of the production of this cytokine by thalidomide treatment. Similarly, interleukin-10 also did not mediate the analgesic effect of thalidomide in carrageenin-induced mechanical hypernociception in rats (Ribeiro et al., 2000b).

It seems that the endogenous opioid is also not involved in the anti-nociceptive effect of thalidomide because naloxone, an opioid receptor antagonist administered to the animals prior to thalidomide, did not affect its analgesic effect.

It is possible that articular edema, rather than articular pain, may have caused the lack of joint mobility that we recorded as articular incapacitation and that thalidomide could be inhibiting the edema and by consequence the articular incapacitation. We had not tested the effect of thalidomide upon joint swelling, although data from other authors point out that the edema observed with the zymosan intrarticular administration is not responsible for its nociceptive response, since experimental drugs which are iNOS inhibitors, though being able to inhibit articular incapacitation, do not alter local edema (da S Rocha et al., 2002). In concordance with these data, other authors demonstrated that the intra-articular injection of dextran, which promotes non-inflammatory edema, did not elicit articular incapacitation, as measured by the rat knee joint incapacitation test, as in the present study (Tonussi and Ferreira, 1992). These previous studies give us support to suggest that the anti-nociceptive effect of thalidomide is not due to an inhibition of the articular edema related to zymosan intra-articular administration.

Taken together, these findings reinforce the notion that TNF-α has a prominent role in inflammatory joint pain and that thalidomide has an effective analgesic action in this process due to its capacity to inhibit TNF-α production by synovial cells. Thus, thalidomide, or some safer analogue, could be useful in the low-cost treatment of joint pain associated with inflammatory arthropathies.

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