



Cloricromene, a coumarine derivative, protects against collagen-induced arthritis in Lewis rats

*¹Salvatore Cuzzocrea, ²Emanuela Mazzon, ³Carla Bevilacqua, ¹Giuseppina Costantino, ⁴Domenico Britti, ⁵Giuseppe Mazzullo, ¹Angela De Sarro & ¹Achille P. Caputi

¹Institute of Pharmacology, University of Messina, Italy; ²Department of Biomorphology, School of Medicine, University of Messina, Italy; ³Fidia spa, Abano Terme, Padova, Italy; ⁴Department of Veterinary Medicine and Pharmacology University of Messina, Italy and ⁵Institute of Veterinary General Pathology and Pathological Anatomy University of Messina, Italy

1 The aim of the present study was to investigate the effects of cloricromene, a coumarine derivative, in rats subjected to collagen-induced arthritis.

2 Collagen-induced arthritis (CIA) was induced in Lewis rats by an intradermal injection of 100 μ l of the emulsion (containing 100 μ g of bovine type II collagen) (CII) and complete Freund's adjuvant (CFA) at the base of the tail. On day 21, a second injection of CII in CFA was administered.

3 Lewis rats developed an erosive hind paw arthritis when immunized with CII in CFA. Macroscopic clinical evidence of CIA first appeared as peri-articular erythema and oedema in the hind paws. The incidence of CIA was 100% by day 27 in the CII challenged rats and the severity of CIA progressed over a 35-day period with radiographic evaluation revealing focal resorption of bone together with osteophyte formation in the tibiotarsal joint and soft tissue swelling.

4 The histopathology of CIA included erosion of the cartilage at the joint margins. Treatment of rats with cloricromene (10 mg kg⁻¹ i.p. daily) starting at the onset of arthritis (day 23), delayed the development of the clinical signs at days 24–35 and improved histological status in the knee and paw.

5 Immunohistochemical analysis for iNOS, COX-2, nitrotyrosine and for poly (ADP-ribose) synthetase (PARS) revealed a positive staining in inflamed joints from collagen-treated rats. The degree of staining for iNOS, COX-2, nitrotyrosine and PARS were markedly reduced in tissue sections obtained from collagen-treated rats, which had received cloricromene.

6 Radiographic signs of protection against bone resorption and osteophyte formation were present in the joints of cloricromene-treated rat.

7 This study provides the first evidence that cloricromene, a coumarine derivative, attenuates the degree of chronic inflammation and tissue damage associated with collagen-induced arthritis in the rat.

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Abbreviations: ecNOS, constitutive endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; MPO, myeloperoxidase; NO, nitric oxide; NOS, nitric oxide synthase; PARS, poly (ADP-ribose) synthetase; PBS, phosphate-buffer saline; PMN, polymorphonuclear cell

Introduction

Rheumatoid arthritis (RA) is a systemic illness characterized by chronic inflammation of the joints and severe cartilage abnormalities, such as joint space narrowing. Tumour necrosis factor (TNF), interleukin-1 (IL-1), and IL-6 are clearly involved in the arthritic process since all three cytokines are present in synovial fluid and can be detected immunohistochemically in the inflamed rheumatoid synovium (Delauran *et al.*, 1992; Farahat *et al.*, 1993). Furthermore, both local and systemic levels of each cytokine correspond to disease activity (Eastgate *et al.*, 1988; Westacott *et al.*, 1990; Feldmann *et al.*, 1990), and TNF and IL-1 have profound catabolic effects on articular cartilage explants from numerous species (Dingle *et al.*, 1987a; Shinmei *et al.*, 1989).

The spontaneous production of IL-1 by rheumatoid synoviocytes can be inhibited by anti-TNF antibodies (Brennan *et al.*, 1989), suggesting that the activity of TNF

occurs earlier in the cascade than that of IL-1, whereas IL-6 occupies a position later in the cascade, being produced in response to either TNF or IL-1 (Guerne *et al.*, 1989; 1990).

Furthermore, IL-1 induces IL-6 synthesis by chondrocytes and is a cofactor in the IL-1-induced suppression of proteoglycan (PG) synthesis (Nietfeld *et al.*, 1990).

Intra-articular injections of TNF and IL-1 cause an influx of neutrophils into the joint and synovitis similar to that seen in experimental arthritis, but only IL-1 results in marked depletion of the cartilage matrix (Dingle *et al.*, 1987b; Van De Loo & Van Den Berg, 1990; Henderson & Pettipher, 1989; O'byrne *et al.*, 1990). *In vitro*, TNF is also less potent than IL-1 in suppressing PG synthesis in cartilage explants (Saklatvala, 1986; Pratta *et al.*, 1989).

Direct evidence that TNF and IL-1 play a role in the pathogenesis of experimental arthritis has been obtained in animal models in which blocking of the action of these cytokines has been shown to delay the onset of collagen-induced arthritis (CIA), suppress inflammation, and ameliorate cartilage destruction that corresponds to the anti-inflammatory response (Wooley *et al.*, 1993a,b; Van Den

*Author for correspondence at: Institute of Pharmacology, School of Medicine, University of Messina, Torre Biologica – Policlinico Universitario Via C. Valeria – Gazzi – 98100 Messina, Italy; E-mail: salvator@www.unime.it

Berg *et al.*, 1994; Williams *et al.*, 1992; Piguet *et al.*, 1992; Thorbecke *et al.*, 1992).

Cloricromene, originally called AD6, is a coumarine derivative, which produces coronary dilation (Aporti *et al.*, 1978), stimulates prostacyclin production from human cultured endothelial cells (Dejana *et al.*, 1982) and lowers thromboxane B2 synthesis from platelet (Galli *et al.*, 1980). Furthermore cloricromene improves survival rate, decreases plasma myocardial depressant factor accumulation and reduces macrophage TxB2 synthesis in splanchnic artery occlusion shock (Squadrito *et al.*, 1988; Sturniolo *et al.*, 1989; 1991).

Here we investigate the effects of cloricromene on the chronic inflammatory response (arthritis) caused by injection of collagen type II in the rat. In addition, we have investigated the effects of cloricromene on the joint injury (histology), cytokines production, the formation of nitrotyrosine (immunohistochemistry) as well as the increases in PARS activity caused by collagen type II injection in the joints.

Methods

Animals

Male Lewis rats (160–180 g; Charles River; Milan; Italy) were housed in a controlled environment and provided with standard rodent chow and water. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purpose (D.M. 116192) as well as with the EEC regulations (O.J. of E.C. L 3581 12/18/1986).

Induction of collagen-induced arthritis

Bovine type II collagen (CII) was dissolved in 0.01 M acetic acid at a concentration of 2 mg ml⁻¹ by stirring overnight at 4°C. Dissolved CII was frozen at -70°C until use. Complete Freund's adjuvant (CFA) was prepared by the addition of Mycobacterium tuberculosis H37Ra at a concentration of 2 mg ml⁻¹. Before injection, CII was emulsified with an equal volume of CFA. Collagen-induced arthritis was induced as previously described (Szabó *et al.*, 1998). On day 1, rats were injected intradermally at the base of the tail with 100 µl of the emulsion (containing 100 µg of CII). On day 21, a second injection of CII in CFA was administered. In another set of experiments, animals were treated with cloricromene (*n* = 10) (10 mg kg⁻¹, i.p. daily) every 24 h, starting from day 23.

Clinical assessment of CIA

Rats were evaluated daily for arthritis by using a macroscopic scoring system: 0 = no signs of arthritis; 1 = swelling and/or redness of the paw or one digit; 2 = two joints involved; 3 = more than two joints involved; and 4 = severe arthritis of the entire paw and digits (Cuzzocrea *et al.*, 2000). Arthritic index for each rats was calculated by adding the four scores of individual paws. Clinical severity was also determined by quantitating the change in the paw volume using plethysmometry (model 7140; Ugo Basile).

Assessment of arthritis damage

At day 35, animals were sacrificed while they were under anaesthesia, and paws and knees were removed and fixed for

histological examination, which was done by an investigator blinded for the treatment regime. The following morphological criteria were considered: score 0, no damage; score 1, oedema; score 2, inflammatory cell presence; score 3, bone resorption.

Histological examination

For microscopic histological evaluation, paws and knees were removed and fixed in 10% formalin. The paws were then trimmed, placed in decalcifying solution for 24 h, embedded in paraffin, sectioned at 5 µm, stained with trichromic Van Gieson and studied using light microscopy (Dialux 22 Leitz).

Radiography

The rats were anaesthetized with sodium pentobarbital (45 mg kg⁻¹, i.p.). Rats were placed on a radiographic box at a distance of 90 cm from the X-ray source. Radiographic analysis of normal and arthritic hind paws was performed by X-ray machine (Philips X12 Germany) with a 40 kW exposition for 0.01 s. An investigator blinded for the treatment regime performed radiograph score. The following radiograph criteria were considered: score 0, no bone damage; score 1, tissue swelling and oedema; score 2, joint erosion; score 3, bone erosion and osteophyte formation.

Immunohistochemical localization of nitrotyrosine and PARS

Tyrosine nitration, an index of the nitrosylation of proteins by peroxynitrite and/or oxygen-derived free radicals, was determined by immunohistochemistry as previously described (Cuzzocrea *et al.*, 1999a). At day 35, the joint's organs were then trimmed, placed in decalcifying solution for 24 h and 8 µm sections were prepared from paraffin embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% H₂O₂ in 60% methanol for 30 min. The sections were permeabilized with 0.1% Triton X-100 in PBS for 20 min. Non-specific adsorption was minimized by incubating the section in 2% normal goat serum in phosphate buffered saline for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with avidin and biotin. The sections were then incubated overnight with primary anti-nitrotyrosine antibody (1:1000) or anti-poly (ADP-Ribose) synthetase (PARS) antibody (1:500) or with control solutions. Controls included buffer alone or non-specific purified rabbit IgG. Specific labelling was detected with a biotin-conjugated goat anti-rabbit IgG (for nitrotyrosine) or with a biotin-conjugated goat anti-rabbit IgG (for PARS) and avidin-biotin peroxidase complex.

Immunohistochemical localization of iNOS and COX-2

iNOS and COX-2 expressions were determined by immunohistochemistry as previously described (Cuzzocrea *et al.*, 1999b). At day 35, the joint's organs were then trimmed, placed in decalcifying solution for 24 h and 8 µm sections were prepared from paraffin embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% H₂O₂ in 60% methanol for 30 min. The sections were permeabilized with 0.1% Triton X-100 in PBS for 20 min. Non-specific adsorption was minimized by incubating the section in 2% normal goat serum in phosphate buffered saline for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with avidin

and biotin. The sections were then incubated overnight with primary anti-iNOS (1:1000) or with primary anti-COX-2 (1:500) with control solutions. Controls included buffer alone or non-specific purified rabbit IgG. Specific labelling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex.

Malondialdehyde (MDA) measurement

Plasma malondialdehyde (MDA) levels were determined as an indicator of lipid peroxidation (Ohkawa *et al.*, 1979). An aliquot (100 μ l) of the plasma collected at the specified time was added to a reaction mixture containing 200 μ l of 8.1% SDS, 1500 μ l of 20% acetic acid (pH 3.5), 1500 μ l of 0.8% thiobarbituric acid and 700 μ l distilled water. Samples were then heated for 1 h at 95°C and centrifuged at 3000 \times g for 10 min. The absorbance of the supernatant was measured by spectrophotometry at 650 nm.

Measurement of cytokines

TNF α and IL-1 β levels were evaluated in plasma at 35 days after the induction of arthritis. The assay was carried out by using a colorimetric, commercial kit (Calbiochem-Novabiochem Corporation, U.S.A.)

Measurement of nitrite/nitrate

Nitrite + nitrate production, an indicator of NO synthesis, was measured in the plasma samples as previously described (Cuzzocrea *et al.*, 1999a). Briefly, the nitrate in the plasma was first reduced to nitrite by incubation with nitrate reductase (670 mu ml⁻¹) and NADPH (160 μ M) at room temperature for 3 h. The nitrite concentration in the samples was then measured by the Griess reaction, by adding 100 μ l of Griess reagent (0.1% naphthylethylenediamide dihydrochloride in H₂O and 1% sulphanilamide in 5% concentrated H₃PO₄; vol. 1:1) to 100 μ l samples. The optical density at 550 nm (OD₅₅₀) was measured using ELISA microplate reader (SLT - Labinstruments Salzburg, Austria). Nitrate concentrations were calculated by comparison with OD₅₅₀ of standard solutions of DMEM.

Materials

Cloricromene was obtained from Fidia Laboratories (Abano Terme, Italy). Perchloric acid was obtained from Aldrich (Milan, Italy). Primary anti-nitrotyrosine, anti-TNF α and anti-poly (ADP-Ribose) synthetase antibodies were from DBA, Milan, Italy. All other reagents and compounds used were obtained from Sigma Chemical Company (Sigma, Milan, Italy).

Data analysis

All values in the figures and text are expressed as mean \pm standard error of the mean (s.e.mean) of *n* observations. For the *in vivo* studies *n* represents the number of animals studied. In the experiments involving histology or immunohistochemistry, the figures shown are representative of at least three experiments performed on different experimental days. Data sets were examined by one- or two-way analysis of variance, and individual group means were then compared with Student's unpaired *t*-test. For the arthritis studies, Mann-Whitney *U*-test (two-tailed, independent) was used to compare medians of the arthritic indices

(Szabó *et al.*, 1998). A *P*-value less than 0.05 was considered significant.

Results

Day of onset and incidence of CIA in Lewis rats

CIA developed rapidly in rats immunized with CII and clinical signs (periarticular erythema and oedema) of the disease (Figure 1A) first appeared in the hind paws between 24 to 26 days post-challenge. Furthermore, a 100% incidence of CIA was observed by day 27 in CII-immunized rats. Neither the clinical signs nor histopathological features of CIA were observed in rat fore paws during the 28-day evaluation period.

The maximum incidence of CIA in the cloricromene-treated rats during the 35-day study period was 60%, (Figure 1A) (*P* < 0.05).

Kinetics of the clinical progression of CIA in Lewis rats

Hind paw erythema and swelling increased in frequency and severity in a time-dependent mode with maximum arthritis indices of approximately 13 observed between 28 to 25 days postimmunization (Figure 1B). Cloricromene exerted a significant suppression (*P* < 0.01) of the arthritis index between days 25 and 35 post-CII immunization (Figure 1B). There was no macroscopic evidence of either hind paw erythema or oedema in the normal control rats (Figure 1B).

Clinical severity of CIA in Lewis rats

The data in Figure 1C demonstrate a time-dependent increase in hind paw (each value represents the mean values of both hind paws) volume (ml) in rats immunized with CII. Maximum paw volume occurred by day 28 in the CII-immunized rats.

Cloricromene (Figure 1C) significantly (*P* < 0.001) suppressed hind paw swelling from day 24 to 35 post-immunization. A maximal reduction in response hind paw swelling of 55% was observed from day 28 to 35. No increase in hind paw volume over time was observed in normal rat (Figure 1C).

Effect of CIA on body weight

The rate and the absolute gain in body weight were comparable in normal Lewis rats and CII-immunized rats for the first week (Figure 1D). Beginning on day 25, the collagen-challenged rats gained significantly less weight than the normal rats, and this trend continued through day 35. After administration of cloricromene, CII-immunized rats exhibited a significant (*P* < 0.0001) protection on weight loss when compared with the respective control group.

Histopathology of CIA

At day 35, histological evaluation of the paws in the vehicle-treated arthritic animals revealed signs of severe arthritis, with massive mixed (neutrophil, macrophage, and lymphocyte) infiltration. In addition, severe or moderate necrosis and sloughing of the synovium were seen, together with the extension of the inflammation into the adjacent musculature with fibrosis (see Figures 2A and 3A for damage score). In

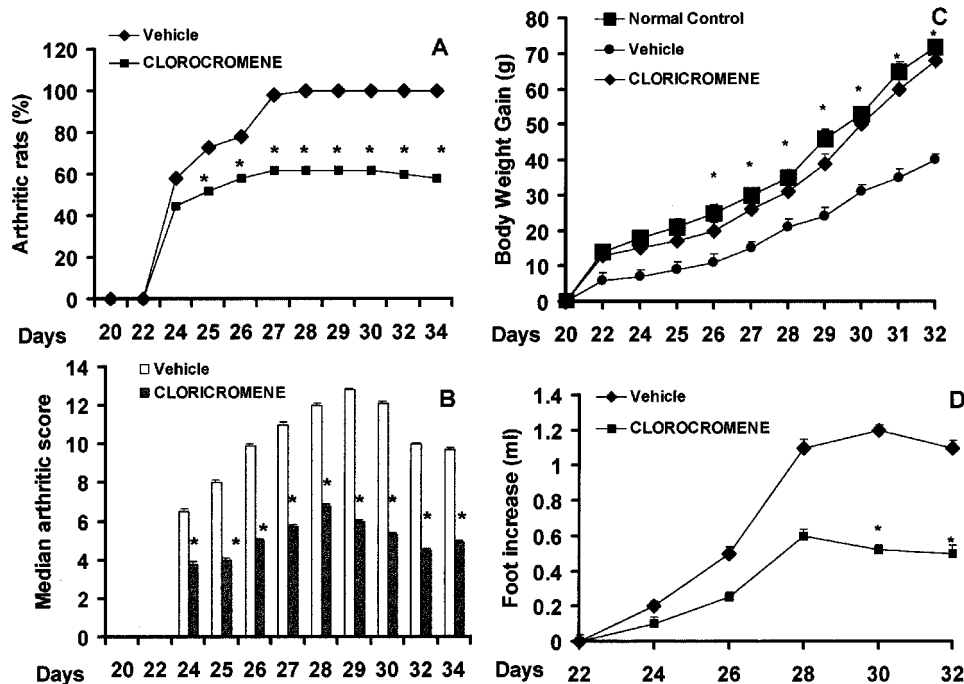


Figure 1 Effect of Cloricromene on the onset, the secondary lesion and on body weight gain in collagen-induced arthritis. The percentage of arthritic rats (rats showing clinical scores of arthritis > 1) are represented (A). There was a significant increase in the arthritic score from day 26 ($P < 0.01$) (B). Beginning on day 25, the collagen-challenged rats gained significantly less weight than the normal rats, and this trend continued through day 35 (C). The swelling in hind paws (D) over time (ml) was measured at 2-day intervals. Cloricromene was able to positively affect in a dose-dependent manner the percentage of arthritic rats, the arthritic score, the weight gain as well as the paw oedema of CIA-immunized rats. Values are means \pm s.e. mean of 10 animals for each group. * $P < 0.01$ versus Control ° $P < 0.01$ versus CIA.

the cloricromene-treated animals, the degree of arthritis was significantly reduced (Figures 2B and 3A).

Radiographic analysis of the effects of cloricromene on CIA

A radiographic examination of hind paws from rats 35 days post CII immunization revealed bone matrix resorption and osteophyte formation at the joint margin (Figure 4A; see Figure 3B for radiograph score). There was no evidence of pathology in normal rats (Figure 3B). Cloricromene markedly reduced the degree of bone resorption, soft tissue swelling and osteophyte formation (Figures 3B and 4B).

Cytokine production

At day 35, the levels of TNF α and IL-1 β were significantly elevated in the plasma from CIA-treated rats (Figure 5). In contrast, the levels of these cytokines were significantly lower in cloricromene-treated rats (Figure 5). No significant cytokine increased was observed in the plasma of sham-operated rats.

Effect of cloricromene on nitric oxide formation

The levels of NO $_x$ were significantly ($P < 0.01$) increased in the plasma from CIA-treated rats (Figure 6). In contrast, the levels of NO $_x$ were significantly lower in the plasma of cloricromene-treated rats (Figure 6). In the joint sections obtained from animals subjected to CIA a positive staining for iNOS was observed (Figure 7A). In contrast, no positive iNOS staining was found in the joints of CIA rats treated with cloricromene (Figure 7B). Staining was absent in control tissue (data not shown).

Effect of cloricromene on COX-2 expression

Immunohistochemical analysis of joint sections obtained from CIA-treated rats revealed a positive staining for COX-2 (Figure 8A). In contrast, no positive COX-2 staining was found in the joints of cloricromene-treated rats (Figure 8B). Staining was absent in tissue obtained from sham-operated control animals (data not shown).

Nitrotyrosine formation and PARS activation

Immunohistochemical analysis of joint sections obtained from rats treated with collagen type II revealed a positive staining for nitrotyrosine, which was primarily localized in synovia portion (Figure 9A). In contrast, no positive nitrotyrosine staining was found in the joint of CIA-treated rats, which has been pre-treated with cloricromene (Figure 9B). Immunohistochemical analysis of joint sections obtained from rats treated with collagen type II also revealed a positive staining for PARS (Figure 10A). In contrast, no specific positive staining for PARS was found in the joint of CIA-treated rats, which had been pre-treated with cloricromene (Figure 10B). Please note that there was no staining for either nitrotyrosine or PARS in joint obtained from sham-operated rats (data not shown).

Effect of cloricromene on MDA levels

At day 35, all vehicle-treated arthritic animals exhibited a substantial increase in the plasma MDA levels (Figure 11). Treatment of rats with cloricromene significantly attenuated the increase in MDA caused by CIA-induced arthritis (Figure 11). No increases in plasma MDA levels were observed with normal rats (Figure 11).

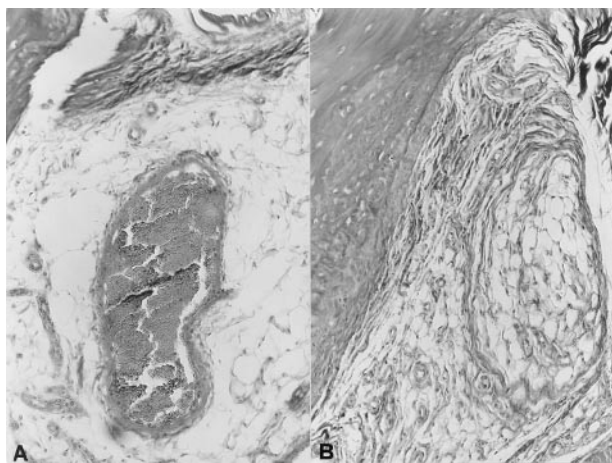


Figure 2 Representative histology of the inflammatory cells infiltration and bone erosion (A) of an arthritic animal. Note the reduction in the degree of inflammatory cells infiltration and bone erosion (B) in the paws of the cloricromene-treated arthritic animals. Original magnification; A,B 100 \times ; Figure is representative of at least three experiments performed on different experimental days.

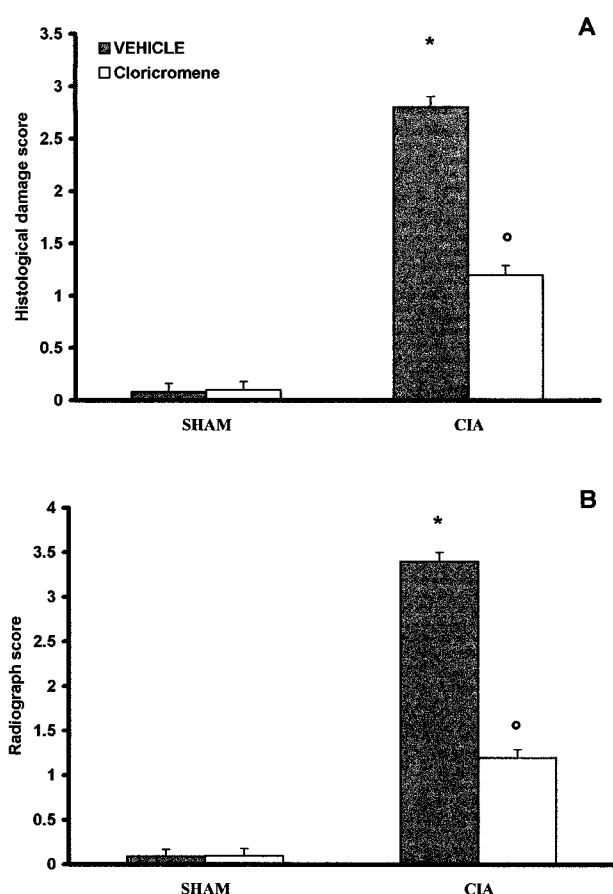


Figure 3 Effect of cloricromene treatment on histological damage score (A), and radiograph score (B). Values are means \pm s.e. mean of 10 animals for each group. * $P < 0.01$ versus Control. ° $P < 0.01$ versus CIA.

Discussion

Collagen-induced arthritis (CIA) is an experimental model of autoimmune disease, which can be induced in mice (Yoo *et al.*, 1988), rats (Cuzzocrea *et al.*, 1999a,b) and primates (Trentham, 1982) by immunization with type II native articular cartilage collagen (CII). The joint pathology

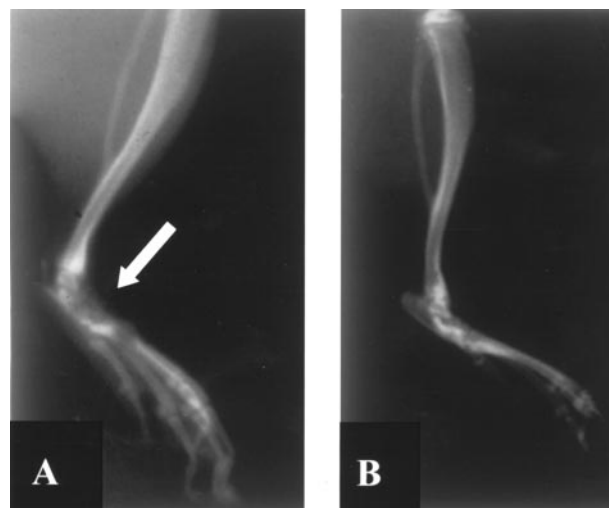


Figure 4 Radiographic progression of CIA in the tibiotarsal joint of rats with CIA. The hind paws from CII-immunized (35 days) rats demonstrated bone resorption (arrow) and joint erosion (A). Cloricromene suppressed joint pathology and soft tissue swelling in the rat hind paw (B). Figure is representative of at least three experiments performed on different experimental days.

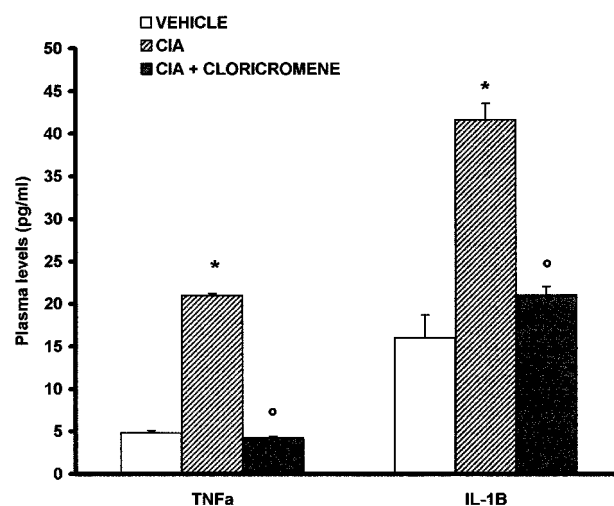


Figure 5 Plasma levels of TNF α and IL-1 β . Cytokine levels were significantly reduced in the plasma from cloricromene-treated rats. Values are means \pm s.e. mean of 10 animals for each group. * $P < 0.01$ versus Control. ° $P < 0.01$ versus CIA.

associated with CIA is similar to the one observed in patients with RA (Stuart *et al.*, 1982a,b). Both cellular and humoral immune responses to CII are involved in the pathogenesis of CIA. For instance, the disease can be transferred by antibodies specific for CII (Wooley *et al.*, 1984; Trentham *et al.*, 1978). Adoptive transfer of lymphoid cells from animals immunized with CII (Holmdahl *et al.*, 1985) and of CII-specific T cell lines and clones (Brahn *et al.*, 1998a) also transmits the disease. Here we demonstrate that cloricromene reduces (i) the development of collagen-induced arthritis, (ii) the infiltration of the joint with PMNs (histology), (iii) the degree of plasma lipid peroxidation, and (iv) the degree of joint injury (histology, radiography) in rats treated with type II collagen. All of these findings support the view that cloricromene attenuates the degree of arthritis and joint injury caused by collagen in the rat. What, then, is the mechanism by which cloricromene protects the joint against this inflammatory injury. Cloricromene is a drug with proved

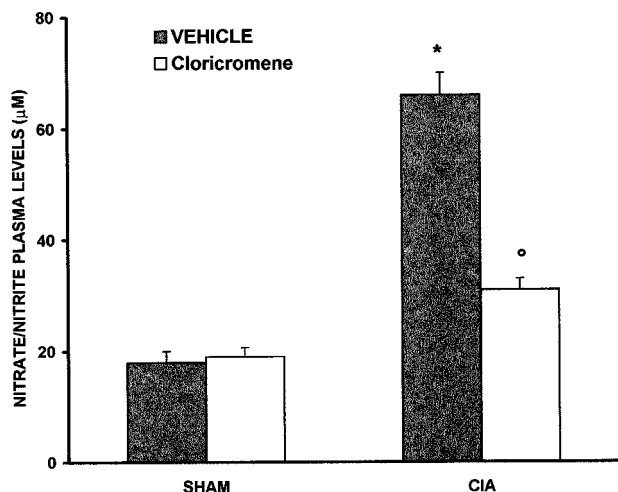


Figure 6 Nitrite and nitrate concentrations in plasma at 35 days after CIA induction. Nitrite and nitrate levels in CIA treated rats were significantly increased *versus* sham group. Cloricromene-treated rats show a significant reduction of the CIA-induced elevation of nitrite and nitrate levels. Values are means \pm s.e. mean of 10 animals for each group. * $P < 0.01$ *versus* Control. ° $P < 0.01$ *versus* CIA.

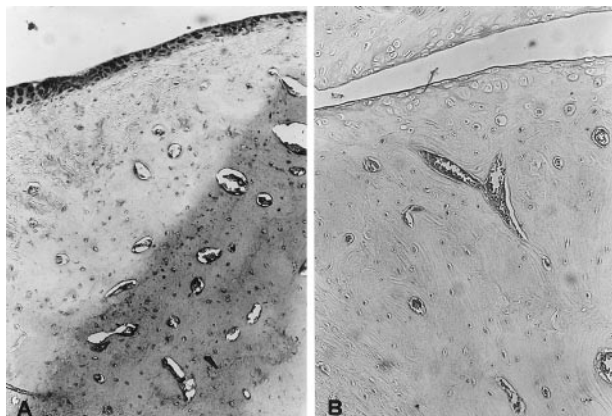


Figure 7 iNOS immunostaining in the paw of a rat at 35 days of collagen-induced arthritis (A). A marked increase in iNOS-like immunoreactivity is evident in the paws in arthritis. There was a marked reduction in the immunostaining in the paw of cloricromene-treated rats (B). Original magnification: $\times 125$. Figure is representative of at least three experiments performed on different experimental days.

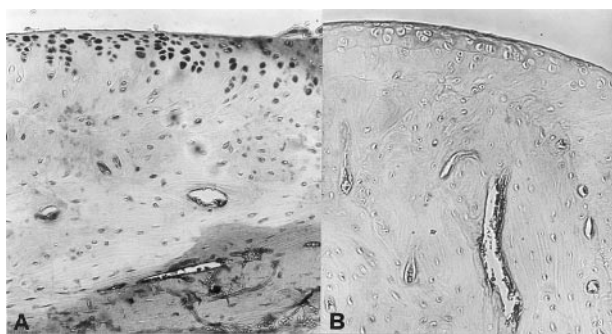


Figure 8 Immunohistochemical analysis, using a specific anti-COX-2 antibody, revealed a positive staining in joints from CIA-treated rats (A). In the joints of the cloricromene-treated (B) no positive staining was found. Original magnification: $\times 125$. Figure is representative of at least three experiments performed on different experimental days.

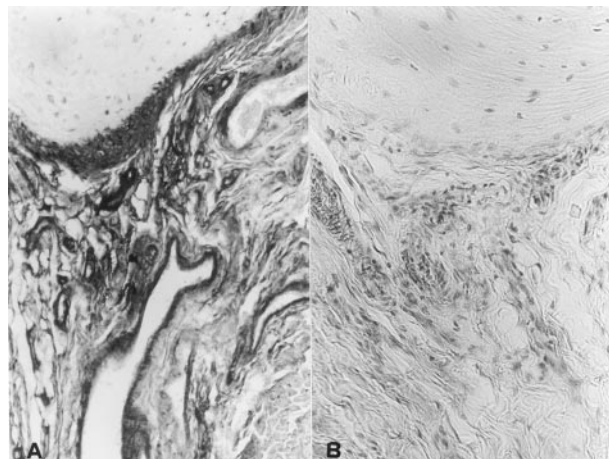


Figure 9 Nitrotyrosine immunostaining in the paw of a rat at 35 days of collagen-induced arthritis (A). A marked increase in nitrotyrosine-like immunoreactivity is evident in the paws in arthritis. There was a marked reduction in the immunostaining in the paw of cloricromene-treated rats (B). Original magnification: $\times 125$. Figure is representative of at least three experiments performed on different experimental days.

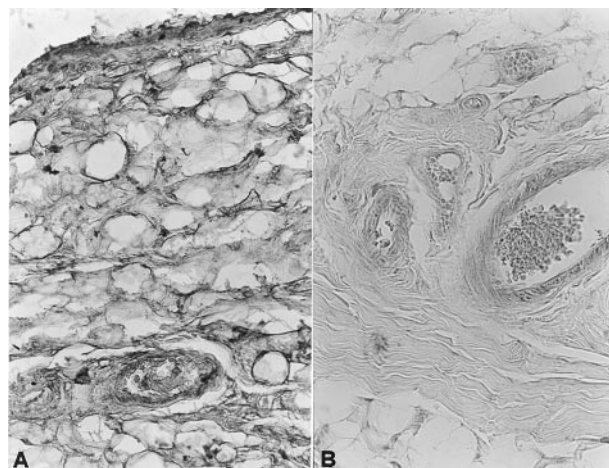


Figure 10 Effect of cloricromene on PARS activity: Staining was absent in control tissue (A). 35 days following collagen-induced arthritis, PARS immunoreactivity was present in the paw from CIA-immunized rats (B). In the paw of cloricromene-treated rats (C), no positive staining was found. Oral magnification: $\times 125$. Figure is representative of at least three experiments performed on different experimental days.

efficacy in several models of experimental shock (Squadrito *et al.*, 1988; Sturniolo *et al.*, 1989; 1991), but the effect of cloricromene in CIA had not yet been investigated. Cloricromene, in fact, possesses interesting properties, which might be relevant to $\text{TNF-}\alpha$ inhibition. As a matter of fact, this coumarine derivative increases macrophage phagocytosis impaired by an experimental model of shock (Squadrito *et al.*, 1992), and reduces thromboxane B2 in macrophage supernatants of splanchnic artery occlusion-shocked rats (Sturniolo *et al.*, 1991), thus indicating that the drug may interfere with primed mononuclear phagocytes. Cloricromene has also been previously reported to inhibit cyclic nucleotide phosphodiesterases in human platelets, but the effect on cyclic AMP-specific phosphodiesterase was present at doses, which were higher than those used in the present study (Hakim *et al.*, 1988; Kunkel *et al.*, 1986). There is evidence that the pro-inflammatory cytokines $\text{TNF-}\alpha$ and IL-1 help to propagate

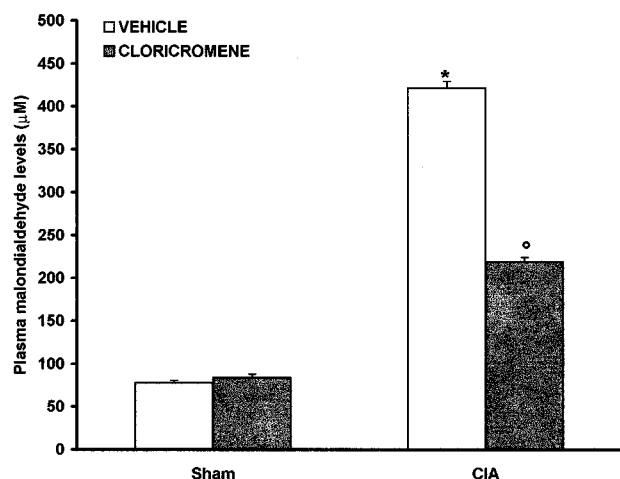


Figure 11 Effect of cloricromene on malondialdehyde levels in the plasma: Malondialdehyde (MDA) levels in the plasma of CII-immunized rats killed at 35 days. MDA levels were significantly increased in the plasma of the CII-immunized rats in comparison to sham rats (* $P < 0.01$). Cloricromene reduced the CIA increase in MDA levels. Values are means \pm s.e. mean of 10 rats for each group. * $P < 0.01$ versus Control. ° $P < 0.01$ versus CIA.

the extension of a local or systemic inflammatory process (Deleuran *et al.*, 1992; Westacott *et al.*, 1990; Feldmann *et al.*, 1990; Shinmei *et al.*, 1989). We confirm here that the inflammatory process (collagen-induced arthritis) leads to a substantial increase in the levels of both TNF α and IL-1 in the plasma. Interestingly, the levels of these two pro-inflammatory cytokines are significantly lower in the plasma obtained from animals, which were treated with cloricromene. These findings confirm that the production of IL-1 during CIA can be inhibited by drugs that inhibit TNF formation, suggesting, in agreement with recent studies (Brennan *et al.*, 1989), that the activity of TNF occurs earlier in the cascade than that of IL-1 which occupies a position later in the cascade (Guerne *et al.*, 1989; 1990). In addition, recent findings (Zatta & Bevilacqua, 1999) have shown that cloricromene can also selectively inhibit PMS function, depending on its concentration. This selectivity may explain the documented activity of this compound in several models of ischaemia – reperfusion and shock.

There is a large amount of evidence that the production of ROS such as hydrogen peroxide, superoxide and hydroxyl radicals at the site of inflammation contributes to tissue damage (Cuzzocrea *et al.*, 1998a; 1999a; Oyanagui, 1994; Salvemini *et al.*, 1998). Inhibitors of NOS activity reduce the development of arthritis and these findings support a role for NO in the pathophysiology associated with this model of inflammation (Brahn *et al.*, 1998b; Ialenti *et al.*, 1993). We demonstrate here that the formation of nitrite and nitrate (metabolites of NO in water) caused by CIA is reduced in cloricromene-treated rats. The induction of iNOS caused e.g. by injection of endotoxin in rodents *in vivo* is mediated by endogenous TNF α , as polyclonal antibodies against this cytokine abolish the induction of iNOS caused by endotoxin in the rat (Thiemermann *et al.*, 1993). Like TNF α , endogenous IL-1 also plays an important role in the induction of iNOS, as the endogenous IL-1 receptor antagonist also reduces the degree of iNOS induction caused by LPS in rodent's (Szabó *et al.*, 1993). As the levels of TNF α and IL-1 are significantly lower in the plasma obtained from cloricromene-treated rats, we propose that the attenuation of the induction of iNOS protein observed in rats, which

are treated with cloricromene is secondary to a reduced formation of endogenous TNF α and IL-1. TNF α and IL-1. Like iNOS, the expression of COX-2 is also mediated by TNF α and IL-1. As the levels of TNF α and IL-1 as well as the induction of iNOS are significantly lower in the plasma obtained from cloricromene-treated rats, it is not surprising that the degree of COX-2 induction is also significantly attenuated in the joints of cloricromene-treated rats. There is good evidence in this and in other models of inflammation that an enhanced formation of prostanoids following the induction of COX-2 contributes to the pathophysiology of local and systemic inflammation (Salvemini *et al.*, 1995; Anderson *et al.*, 1996) and also that selective inhibitors of COX-2 exert potent anti-inflammatory effects (Anderson *et al.*, 1996; Mitchell *et al.*, 1993; Harada *et al.*, 1996).

In addition to prostaglandins and NO, peroxynitrite is also generated in CIA-induced inflammation (Szabó *et al.*, 1998; Cuzzocrea *et al.*, 2000). The biological activity and decomposition of peroxynitrite is very much dependent on the cellular or chemical environment (presence of proteins, thiols, glucose, the ratio of NO and superoxide, carbon dioxide levels and other factors), and these factors influence its toxic potential (Beckman *et al.*, 1990; Rubbo *et al.*, 1994). We demonstrate here that CIA leads to a substantial increase in the degree of nitrosylation of proteins in the joint. In contrast, the degree of staining for nitrotyrosine was significantly reduced in cloricromene-treated rats. Nitrotyrosine formation, along with its detection by immunostaining, was initially proposed as a relatively specific marker for the detection of the endogenous formation (footprint) of peroxynitrite (Beckman, 1996). There is, however, recent evidence that certain other reactions can also induce tyrosine nitration; e.g., the reaction of nitrite with hypochlorous acid and the reaction of myeloperoxidase with hydrogen peroxide can lead to the formation of nitrotyrosine (Eiserich *et al.*, 1998). Increased nitrotyrosine staining is considered, therefore, as an indication of 'increased nitrosative stress' rather than a specific marker of the generation of peroxynitrite.

ROS and peroxynitrite produce cellular injury and necrosis *via* several mechanisms including peroxidation of membrane lipids, protein denaturation and DNA damage. ROS produce strand breaks in DNA which triggers energy-consuming DNA repair mechanisms and activates the nuclear enzyme PARS resulting in the depletion of its substrate NAD *in vitro* and a reduction in the rate of glycolysis. As NAD functions as a cofactor in glycolysis and the tricarboxylic acid cycle, NAD depletion leads to a rapid fall in intracellular ATP. This process has been termed 'the PARS Suicide Hypothesis' (Szabó *et al.*, 1997; 1998; Cuzzocrea *et al.*, 1998b; 2000). There is recent evidence that the activation of PARS may also play an important role in inflammation (Szabó *et al.*, 1997; 1998; Cuzzocrea *et al.*, 1998b; 2000). We demonstrate here that cloricromene attenuates the increase in PARS activity caused by collagen in the joint.

In conclusion, this study demonstrates that the coumarine derivative cloricromene attenuates the arthritis caused by collagen administration in the rat. We speculate that the observed anti-inflammatory effects of cloricromene may be dependent upon a combination of the following pharmacological properties of this agent: (1) Cloricromene inhibit TNF- α , which would prevent the production of IL-1 β . This, in turn, prevents the expression of iNOS and COX-2 protein, and ultimately the degree of peroxynitrite formation and tissue injury. (2) Cloricromene appears to prevent the activation of PARS. Finally, our findings suggest that interventions that may reduce the generation or the effects

of TNF- α may be useful in conditions associated with local or systemic inflammation.

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