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Leukotriene B_4 plays a critical role in the progression of collagen-induced arthritis

R. J. GRIFFITHS*, E. R. PETTIPHER, K. KOCH, C. A. FARRELL, R. BRESLOW, M. J. CONKLYN, M. A. SMITH, B. C. HACKMAN, D. J. WIMBERLY, A. J. MILICI, D. N. SCAMPOLI, J. B. CHENG, J. S. PILLAR, C. J. PAZOLES, N. S. DOHERTY, L. S. MELVIN, L. A. REITER, M. S. BIGGARS, F. C. FALKNER, D. Y. MITCHELL, T. E. LISTON, AND H. J. SHOWELL

Central Research Division, Pfizer Inc., Groton, CT 06340

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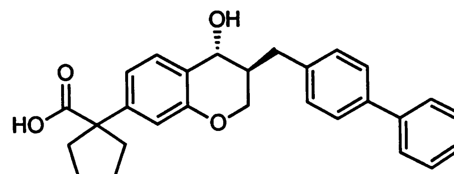
ABSTRACT **Leukotriene B_4 (LTB_4) is a product of the 5-lipoxygenase pathway of arachidonic acid metabolism. LTB_4 is a potent chemotactic factor for neutrophils and has been postulated to play an important role in a variety of pathological conditions including rheumatoid arthritis (RA), psoriasis, and inflammatory bowel disease. The role of LTB_4 in such diseases has not yet been defined but in this paper we provide direct evidence that LTB_4 plays a critical role in a murine model of RA. CP-105,696, (+)-1-(3*S*,4*R*)-[3-(4-phenylbenzyl)-4-hydroxychroman-7-yl]cyclopentane carboxylic acid, is an LTB_4 receptor antagonist that inhibits LTB_4 binding to human neutrophil membranes with an IC_{50} of 3.7 nM and inhibits LTB_4-induced chemotaxis of these cells with an IC_{50} of 5.2 nM. CP-105,696 inhibits LTB_4-induced neutrophil influx in mouse skin when administered orally with an ED_{50} of 4.2 mg/kg. CP-105,696 had a dramatic effect on both the clinical symptoms and histological changes of murine collagen-induced arthritis when administered at doses of 0.3–10 mg/kg. Inhibition was not associated with suppression of the humoral immune response to collagen and was equally effective if drug treatment was commenced just prior to the onset of arthritis or throughout the experiment. These results suggest that LTB_4 receptor antagonists may be effective therapeutic agents for the treatment of RA.**

Rheumatoid arthritis (RA) is a chronic inflammatory polyarthritis that is inadequately treated with currently available drugs (1). One potential strategy to better treat this disease is to reduce the influx of leukocytes into the joint, since recent studies have shown that the extent of neutrophil infiltration into the joints of RA patients precedes clinical signs of inflammation and is predictive of pain (2). There are a number of mediators of the inflammatory response that could potentially be responsible for neutrophil accumulation, but leukotriene B_4 (LTB_4) is an attractive target since it is a potent chemotactic agent for human neutrophils (3), is produced in large amounts by these cells, and is found in the synovial fluid of patients with RA (4).

In this paper, we describe experiments to assess the role of LTB_4 in a murine model of RA, collagen-induced arthritis. The immunological and histological features of this model resemble those seen in RA patients. The strategy we employed was to use a LTB_4 receptor antagonist to block the biological effects of endogenously produced LTB_4. Several potent and selective LTB_4 receptor antagonists, with a variety of structural types, have been reported (reviewed in ref. 5). However, there are no data on the efficacy of these agents in models of arthritis. CP-105,696, (+)-1-(3*S*,4*R*)-[3-(4-phenylbenzyl)-4-hydroxychroman-7-yl]cyclopentane carboxylic acid, is a newly discovered LTB_4 receptor antagonist that has a high affinity



for human and mouse LTB_4 receptors and has a long plasma half-life in the mouse, which allows the maintenance of pharmacologically relevant concentrations of the drug with a once daily dosing protocol. Here we report the use of CP-105,696 to demonstrate the importance of LTB_4 as a critical mediator in the pathogenesis of murine collagen-induced arthritis.

MATERIALS AND METHODS

***In Vitro* LTB_4 Receptor Ligand Binding Assays.** The procedure for [^{3}H]LTB_4 binding was adapted from the method of Cheng and co-workers (6). Binding was performed in 150 μl in a buffer containing 50 mM Tris·HCl (pH 7.3), 10 mM $MgCl_2$, 9% methanol, 0.7 nM [^{3}H]LTB_4 (5920–7400 GBq/mmol; New England Nuclear), and either 0.83 mg (murine spleen) or 0.13 mg (human neutrophil) of membrane per ml. Unlabeled LTB_4 was added at a concentration of 5 μM to determine nonspecific binding. Incubations were carried out in microtiter plates at 4°C for 30 min and the bound ligand was separated from the free ligand with a Betaplate apparatus (Pharmacia LKB) with double-thickness glass fiber filter mats.

***In Vitro* Chemotaxis Assay.** Chemotaxis assays were performed as described by Harvath and co-workers (7). Neutrophils were isolated according to the procedure of Ferrante and Thong (8); resuspended at a density of 3×10^6 cells per ml in Hanks' balanced salt solution containing calcium, magnesium, and 2 mg of bovine serum albumin (BSA) per ml; and placed in the upper wells of a multiwell chemotaxis chamber (Neuroprobe, Cabin John, MD). Serial dilutions of CP-105,696 were present in both wells of the chemotaxis chamber, and LTB_4 (5 nM) was present in the bottom chamber. After a 1-hr incubation at 37°C the cellulose nitrate filters (pore size, 3 μm) were removed from the chambers and fixed, stained, and assayed. The total number of cells (observed microscopically at ×400) migrating from 20 μm beneath the monolayer to the leading front (measured at 20-μm intervals) were summed and provided an index of cell migration.

***In Vitro* Adhesion Molecule Expression Assay.** Human neutrophils were isolated as described above and resuspended at a density of $1.5–3.0 \times 10^7$ cells per ml in phosphate-buffered saline containing 10 mM EDTA. In test tubes, serial dilutions

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Abbreviations: LTB_4, leukotriene B_4; RA, rheumatoid arthritis; IL-1α, interleukin 1α.
*To whom reprint requests should be addressed.

of CP-105,696 were added to the cells and incubated for 5 min at 37°C followed by LTB_4 to achieve a final concentration of 5 nM. After a further incubation for 10 min at 37°C, cells were washed and then labeled with fluorescein isothiocyanate-labeled anti-CD11b (Gen-Trak Systems, Framingham, MA), washed and then contaminating red blood cells were lysed with fluorescence-activated cell sorting lysing solution (Becton Dickinson), rewashed, and then resuspended for quantification of fluorescent staining with a Becton Dickinson FACScan flow cytometer.

LTB_4-Induced Neutrophil Influx in Mouse Skin. Male BALB/c mice were anesthetized with methoxyflurane and injected intradermally with LTB_4 (100 ng in 20 μl of saline containing 1 mg of BSA per ml), and neutrophil infiltration was measured after 4 hr by the myeloperoxidase content of the skin. Known numbers of mouse neutrophils (harvested from the peritoneal cavity after injection of oyster glycogen) were included in each assay as a standard curve and data are expressed as numbers of neutrophil equivalents per site. Drug was dosed orally 1 hr prior to injection of LTB_4 (9).

Induction and Assessment of Collagen-Induced Arthritis. Male DBA/1LacJ (9–13 weeks old) mice were immunized at the base of the tail with 100 μg of chicken type II collagen in Freund's complete adjuvant on days 0 and 21. Severity of the symptoms of arthritis was assessed by inspection of the paws (0 = normal paw, 1 = swelling and/or redness of one toe or finger joint, 2 = two or more joints involved, and 3 = severe arthritis in the entire paw; maximum score for each animal = 12). In some experiments, administration of interleukin 1α (IL-1α) was used to cause a severe flare of the arthritis; 0.3 μg of recombinant murine IL-1α, diluted in phosphate-buffered saline containing 1 mg of BSA per ml, was administered s.c. on the days indicated. This protocol causes all of the control animals to exhibit a severe form of the disease and is valuable for pharmacological studies since it is more reproducible and allows the number of animals in each group to be reduced. Changes in body weight over the course of the experiment were also monitored. Nonimmunized mice treated with IL-1 lose body weight, but by day 7 this has returned to normal. The immunized mice show a more profound and persistent fall in body weight so that at day 7 the weight loss changes are seen only in arthritic mice. CP-105,696 or vehicle [methylcellulose in water (5 mg/ml)] were administered orally in a dose volume of 10 ml/kg once daily. Treatment commenced either the day before the first immunization or the day before the first injection of IL-1. Groups of 15–20 mice were used for the standard protocol, and groups of 7–9 mice were used for the IL-1-stimulated protocol. Each experiment has been repeated on at least three occasions.

Histopathology. Knee joints were decalcified in Kristensen's solution, embedded in Paraplast plus, sectioned, and stained

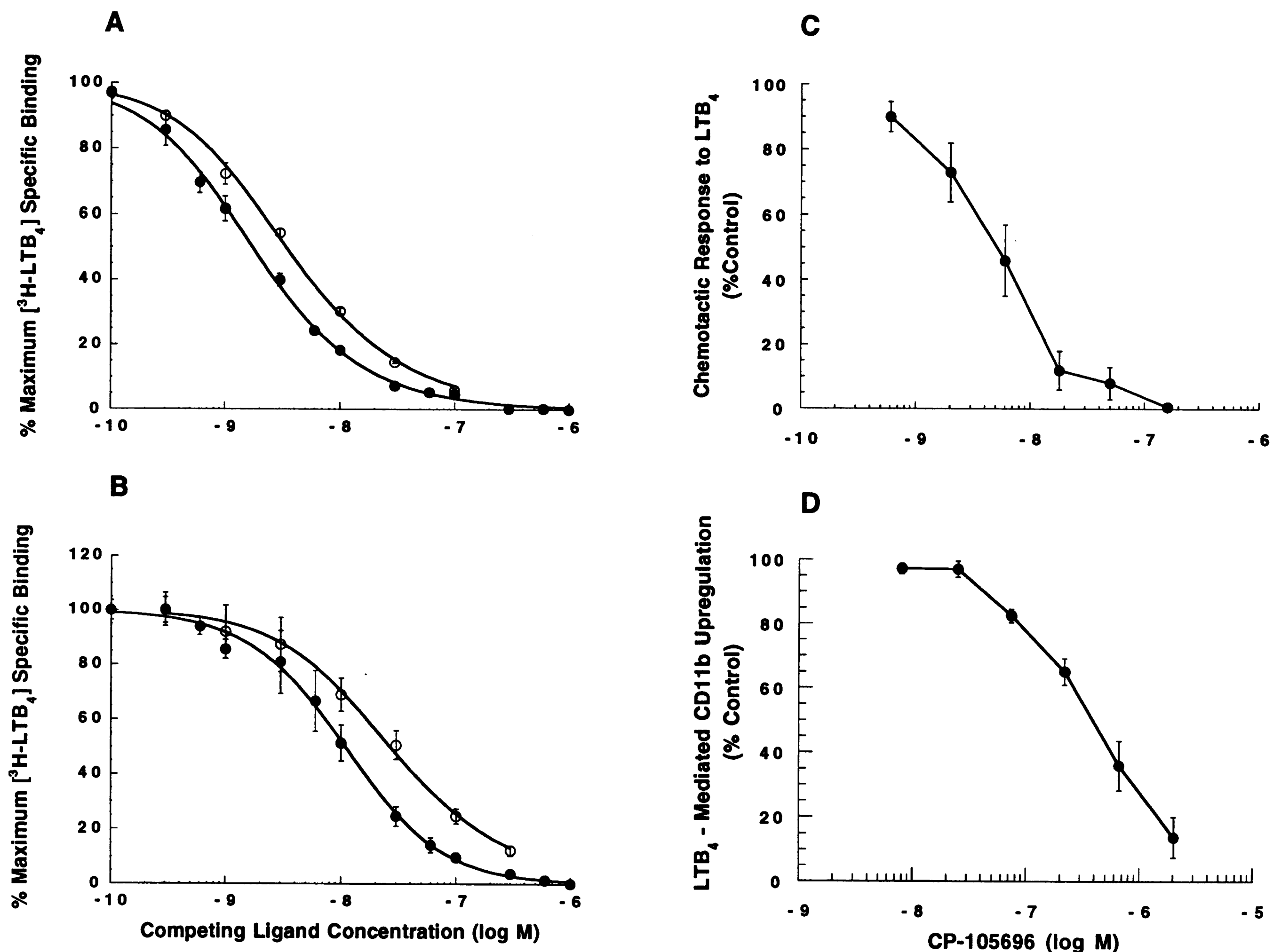

FIG. 1. *In vitro* antagonism of LTB_4 receptors by CP-105,696. (*A* and *B*) Displacement of specific [^{3}H]LTB_4 binding to human neutrophil (*A*) and murine (C3H/HEJ) spleen (*B*) membranes by LTB_4 (●) and CP-105,696 (○). Values depicted are means ± SEM of three independent experiments. (*C*) Inhibition of LTB_4-mediated human neutrophil chemotaxis by CP-105,696. Values depicted are means ± SEM of five independent experiments. (*D*) Inhibition of LTB_4-mediated upregulation of the α subunit of the β_2-integrin CD11b/CD18 by CP-105,696. Values depicted are means ± SEM of four independent experiments.

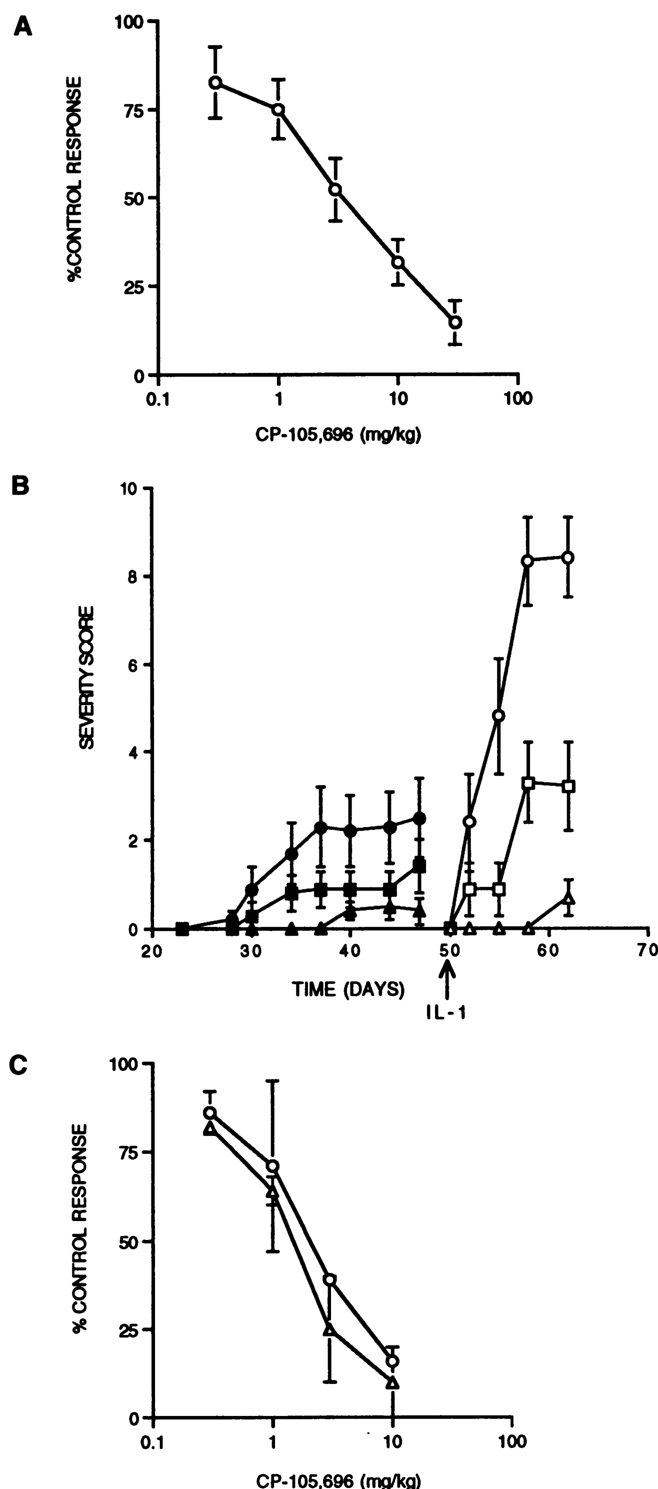

FIG. 2. *In vivo* effects of CP-105,696 in murine models of inflammation. (*A*) Inhibition of LTB_4-mediated neutrophil infiltration by CP-105,696. Values depicted are means ± SEM of three independent experiments. CP-105,696 doses of 3 mg/kg and greater produced statistically significant effects compared to controls ($P < 0.05$). (*B* and *C*) Inhibition of the symptoms of collagen-induced arthritis by CP-105,696. (*B*) Mice were dosed with vehicle (●, ○), 1 mg (■, □), or 10 mg (▲, △) of CP-105,696 per kg daily throughout the experiment. Any animal that had not developed arthritis by day 50 was given an s.c. injection of recombinant murine IL-1α (0.3 μg) on days 50 and 51. Results show a typical experiment representative of four performed. Effects of both doses of CP-105,696 were statistically significant compared to control ($P < 0.05$). (*C*) Drug treatment commenced on day 25, IL-1 was administered on days 26 and 27, and severity of arthritis (○) and change in body weight (△) were measured on day 34.

with Safranin O; approximately matched sections were examined by light microscopy on a Nikon FXA microscope. Quantitation of the proteoglycan staining in the femoral condyle was performed as described (10).

Anti-Collagen Antibody Levels. IgG antibody levels against the immunizing antigen were measured by standard ELISA methodology. Microtiter plates were coated with 2.5 μg of chicken collagen per well, blocked with BSA, and then incubated with dilutions of the test sera. An alkaline phosphatase rabbit anti-mouse IgG was then added, followed by substrate (*p*-nitrophenyl phosphate). Results are expressed as the reciprocal of the dilution causing 50% of the maximum absorbance change at 405 nm.

Statistics. Results of the *in vivo* experiments were analyzed by Student's *t* test with a Bonferroni correction factor for multiple comparisons.

RESULTS

Structure. The structure of CP-105,696 is shown in Fig. 1.

***In Vitro* Activity in Ligand Binding Assays.** CP-105,696 is a highly potent inhibitor of the binding of $[^3H]LTB_4$ to human neutrophil and mouse spleen membranes with IC_{50} values at 3.7 and 30.3 nM, respectively (Fig. 1 *A* and *B*). Respective IC_{50} values for LTB_4 obtained in the same ligand binding assays (Fig. 1 *A* and *B*) were 2.1 nM (human neutrophil) and 10.3 nM (mouse spleen).

***In Vitro* Activity in Functional Assays.** LTB_4-induced chemotaxis and upregulation of the adhesion molecule CD11b in human neutrophils have been used to demonstrate the antagonist activity of CP-105,696 in functional assays. Chemotaxis (a function mediated by the high-affinity form of the LTB_4 receptor) was inhibited by CP-105,696 with an IC_{50} value of 5.2 nM (Fig. 1*C*) and adhesion molecule upregulation (a function mediated by the low-affinity form of the receptor) with an IC_{50} of 430 nM (Fig. 1*D*). CP-105,696 did not demonstrate any agonist activity in these assays, and at 10 μM it did not inhibit chemotaxis in response to C5a, platelet activating factor, or IL-8 (data not shown), demonstrating the selectivity of the compound.

***In Vivo* Inhibition of LTB_4-Induced Neutrophil Infiltration.** To demonstrate the ability of CP-105,696 to inhibit the biological activity of LTB_4 *in vivo*, LTB_4 was injected intradermally into mouse skin and neutrophil accumulation was measured. Oral administration of CP-105,696 inhibited this response with an ED_{50} value of 4.2 mg/kg (Fig. 2*A*). To demonstrate the selectivity of CP-105,696, it was tested for the ability to inhibit IL-1-induced neutrophil accumulation. Neutrophil equivalents (as measured by myeloperoxidase content of the skin) at IL-1-injected sites in vehicle-treated mice were $7.7 \pm 1.3 \times 10^5$. In mice treated with 100 mg of CP-105,696 per kg the corresponding value was $11.2 \pm 1.1 \times 10^5$, which was not significantly different from the response in vehicle-treated mice. In addition, CP-105,696 has been tested for the ability to inhibit zymosan-stimulated leukotriene and prostaglandin production in the mouse peritoneal cavity. Doses of up to 30 mg/kg do not affect the production of either of these products (data not shown).

Inhibition of the Development of Collagen Arthritis. The pharmacokinetic profile of CP-105,696 in the mouse was studied before commencing experiments in chronic models. The drug is well absorbed after oral administration (>50%) and has a plasma elimination half-life of 33 hr after i.v. administration. This means that a single daily dosing protocol

Values depicted are means ± SEM of three independent experiments. CP-105,696 doses of 3 mg/kg and greater produced statistically significant effects compared to controls ($P < 0.05$). Each experimental group consisted of 7–20 mice. All experiments with animals were in accordance with institutional guidelines.

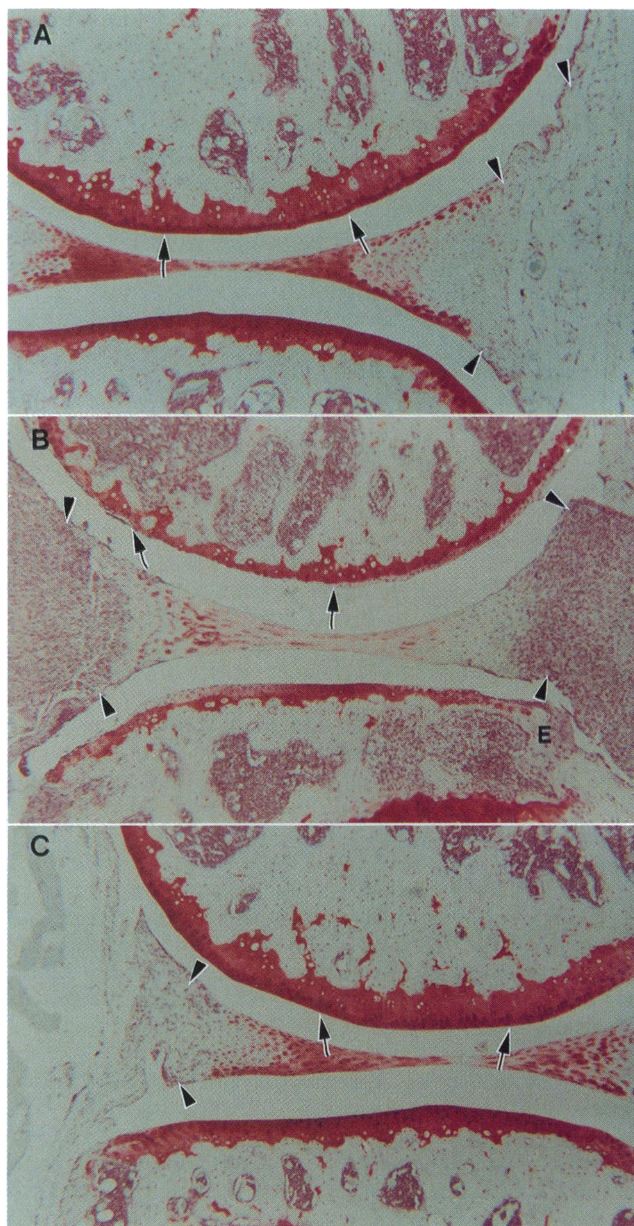

FIG. 3. Effect of CP-105,696 on histological changes in the joints of mice with collagen-induced arthritis. (*A*) Sham-immunized animal treated with vehicle showing the normal articular cartilage proteoglycan staining (arrows) and typical cellularity of the subsynovial connective tissue (arrowheads). (*B*) In contrast, in the collagen-immunized mice treated with vehicle there was destruction of the articular cartilage (arrows), erosion of bone (E), and a massive influx of inflammatory cells into the subsynovial connective tissue (arrowheads). (*C*) In immunized mice treated with 10 mg of CP-105,696 per kg articular cartilage (arrows) and subsynovial connective tissue (arrowheads) were indistinguishable from controls. (×50.)

can be used to provide adequate plasma levels to antagonize the LTB_4 receptor for 24 hr per day.

The incidence of the disease in this model varies (in our hands, the proportion of mice showing any sign of arthritis varies from 60% to 90%) and the severity of the disease within a particular group of mice can also be extremely variable. Administration of the cytokine IL-1 as an adjunct in this model increases the incidence and severity of the disease, which is useful for pharmacological studies (11). The mechanism of this exacerbation is not clear but it may relate to the neutrophilia this cytokine causes. In the experiment shown in Fig. 2*B*, daily dosing of CP-105,696 starting the day before immunization decreased the severity of arthritis in immunized animals at doses of 1 and 10 mg/kg. There was no evidence of any toxic effects of the compound even after this extensive period of dosing. Fifty days into the experiment, animals that had not demonstrated any sign of arthritis were given two s.c. injections of IL-1 24 hr apart. This caused all the animals in the vehicle-treated group to rapidly develop a severe arthritis. However, the animals treated with 1 mg of CP-105,696 per kg developed a much less severe form of the disease, and at 10 mg/kg the disease was almost completely suppressed (Fig. 2*B*). CP-105,696 did not affect IgG antibody titers to type II collagen in this model [titer in vehicle-treated mice was $1/(3732 \pm 252)$ compared to $1/(3818 \pm 295)$ in mice treated with CP-105,696 at a dose of 10 mg/kg throughout the experiment], suggesting that it does not interfere with the humoral immune response to the immunizing antigen.

Inhibition of the Progression of Collagen Arthritis. The activity of CP-105,696 in this model was explored further by using the IL-1-exacerbated protocol. We found that if dosing of CP-105,696 was started 1 day before the first injection of IL-1 (day 26 after immunization) it was just as efficacious as when dosed throughout the experiment (Fig. 2*C*). Vehicle-treated animals lose body weight as the arthritis develops. Nonimmunized mice treated with IL-1 lose body weight, but by day 7 this has returned to normal. The immunized mice show a more profound and persistent fall in body weight so that at day 7 the weight loss changes are seen only in arthritic mice. Body weight loss at early times after IL-1 administration was not affected by CP-105,696. However, the weight loss observed at day 7 was reduced, in parallel with the inhibition of arthritis development (Fig. 2*C*). This suggests that the persistent weight loss seen in the arthritic mice may be secondary to an impairment in feeding due to the pain and disability of the arthritis.

A histological examination of the joints of vehicle-treated animals revealed a massive inflammatory cell influx with extensive cartilage and bone damage. Although not obviously affected by macroscopic inspection, the knee joints proved to be severely affected when assessed histologically. Since reproducible sections can be more easily prepared from this joint, it was used for quantitative histological studies. Treatment with CP-105,696 prevented not only the inflammatory cell influx but also the cartilage and bone destruction (Fig. 3). Quantitation of the proteoglycan content of the femoral condyle by image analysis confirms this observation. The extent of proteoglycan staining in the articular cartilage of the femoral condyle was expressed as a percentage of that in the growth plate of each animal, which acted as an internal control. In normal knees, this value was 65.9% ± 3.4%, which decreased to 26.6% ± 6.8% in immunized mice treated with vehicle. In mice treated with CP-105,696 (10 mg/kg), this was increased to 48.3% ± 4.1% ($P < 0.05$ compared to vehicle group, mean ± SEM; $n = 3$ experiments).

DISCUSSION

Although there is no perfect animal model of RA, murine collagen-induced arthritis does share certain features with the human disease. Neutrophil infiltration into the joints is a prominent feature of mice with collagen-induced arthritis, and it has been shown that antibody depletion of circulating neutrophils is antiinflammatory in this model (12, 13). Clinical data have shown that neutrophil infiltration into the joints of patients with RA is predictive of pain and precedes clinical signs of inflammation (2). The histological features of this model closely approximate those observed in rheumatoid joints, and a proportion of patients with RA display humoral and cellular immunity to type II collagen. Furthermore, the importance of immunity to type II collagen in the pathogenesis of RA has recently been demonstrated by the finding that

ingestion of type II collagen can suppress disease, presumably by inducing a state of immunological tolerance (14). Several recent studies have shown that biological reagents that neutralize the effects of the cytokines tumor necrosis factor (TNF) (15–17) and IL-1 (18–20) are efficacious in this murine model of RA. These experiments led to trials of anti-TNF antibodies in RA patients, where striking therapeutic efficacy has been reported (21). The studies reported here directly implicate LTB_4 as a critical mediator of inflammatory arthritis and suggest that trials of such agents in RA are warranted. Clearly there would be a great advantage in terms of cost and convenience in treating human subjects with an orally active, low molecular weight agent rather than i.v. administration of an antibody or soluble receptor.

CP-105,696 inhibits disease expression in this model by targeting the effector arm of the immune/inflammatory response. This is supported by the fact that the drug had no effect on antibody levels to collagen, and it was equally efficacious when dosed either throughout the experiment, or just prior to disease onset. The efficacy of a LTB_4 antagonist in the standard, non-IL-1-boosted version of this model is at least equivalent if not superior to anticytokine interventions. The fact that the therapeutic effect of CP-105,696 was evident even in immunized animals treated with IL-1 is remarkable, since these animals exhibit a particularly rapid and severe flare response. This may at first sight seem surprising. However, the likely explanation is that LTB_4 exerts its effects distal to those of cytokines such as IL-1 and TNF. This suggests that there is a limited redundancy of mediators of the inflammatory response and holds out the hope that new and effective treatments for chronic inflammatory disease in humans may be on the horizon. Drugs such as CP-105,696 will allow us to test the hypothesis that LTB_4 plays a similar role in human inflammatory joint disease.

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