Collagen-Induced Arthritis as a Model of Hyperalgesia

Functional and Cellular Analysis of the Analgesic Actions of Tumor Necrosis Factor Blockade

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Objective. There is a disparity in the animal models used to study pain in rheumatoid arthritis (RA), which tends to be acute in nature, and models used to assess the pathogenesis of RA. The latter models, like human RA, are lymphocyte-driven and polyarticular. We assessed pain behavior and mechanisms in collagen-induced arthritis (CIA), the model of preclinical arthritis used most commonly in the field of immunology. We then validated the model using anti–tumor necrosis factor (anti-TNF) therapy, which has analgesic effects in models of inflammation as well as in human RA.

Methods. CIA was induced in DBA/1 mice by immunization with type II collagen at the base of the tail. Swelling and mechanical and thermal hyperalgesia were assessed before and for 28 days after the onset of arthritis. Spontaneous behavior was assessed using an automated activity monitor. Glial activity was assessed by glial fibrillary acidic protein expression, and nerve damage was evaluated by activating transcription factor 3 expression. The actions of anti-TNF therapy on nociception were then evaluated.

Results. Arthritis resulted in a decrease in the threshold for thermal and mechanical stimuli, beginning on the day of onset. Decreased spontaneous activity was also observed. A significant increase in the number of hyperplastic spinal cord astrocytes was observed beginning 10 days after the onset of arthritis. Anti-TNF therapy was profoundly analgesic, with an efficacy similar to that of cyclooxygenase 2 inhibition, and reduced astrocyte activity in CIA.

Conclusion. This study shows that the CIA model is suitable for testing not only antiinflammatory but also analgesic drugs for potential use in RA, and highlights the importance of using appropriate disease models to assess relevant pain pathways.

Several animal models are used for the study of inflammation and inflammatory pain. The most commonly used models for the study of inflammatory pain involve injecting mediators of inflammation into the footpad or knee (1). This results in a monarthritis, in which the contralateral limb can be used as an internal control. However, the inflammation in these models is acute and often self limiting in nature, differing from that in arthritis, and does not involve B lymphocytes or T lymphocytes, which are known to be key in the pathogenesis of rheumatoid arthritis (RA). Antigen-induced arthritis, in which mice are immunized with methylated bovine serum albumin, has been used to study pain in arthritis (2). This model is T cell dependent but, unlike RA, is not major histocompatibility complex restricted or B cell dependent. Streptococcal cell wall–induced arthritis has also been used for the study of arthritic pain. However, this model more closely resembles reactive arthritis than human RA.

In contrast to models used to study pain, the animal model that is currently most widely used to study the pathogenesis and treatment of RA is collagen-induced arthritis (CIA) in male DBA/1 mice (3). CIA is induced by immunization with type II collagen in Freund’s complete adjuvant (CFA), which induces an...
autoimmune disease directed against the cartilage in the joints. The model closely resembles human RA in many respects. CIA is a polyarthritic condition characterized by inflammation and destruction of the joints in a T cell– and B cell–specific manner. Despite this being the most widely used rodent model for the study of RA, its use for the study of arthritic pain and treatment with analgesics has not been reported. In addition, changes that occur in the nervous system during the course of CIA have not been well studied, and it is not known whether a similar mechanism of sensitization occurs in monarthritis and polyarthritis conditions (4,5).

The CIA model in the DBA/1 mouse was successfully used to predict the beneficial effects of human tumor necrosis factor α (TNFα) blockade in RA (6,7). Subsequently, it has been shown that cytokines also play a crucial role in both inflammatory and neuropathic pain (8–10). In a recent review article, Verri et al discussed the roles of cytokines and chemokines in inflammatory pain (1). Although most of the evidence for the role of these inflammatory cytokines in inflammation came from CIA, it is interesting that all of the evidence for their role in inflammatory pain came from models of acute inflammation.

Changes in the nociceptive system that occur during acute inflammation are unlikely to be the same as those that occur in chronic immune-mediated diseases such as RA. Our ultimate aim is to identify novel targets for the severe pain associated with RA. Therefore, we wanted to establish whether CIA could be used to assess pain and behavioral changes in mice. Because hyperalgesia has not been fully studied in models of polyarthritis, we used 2 different methods of assessment (11). Standard techniques were used to measure mechanical and thermal hyperalgesia, using von Frey hairs and the Hargreaves test, respectively. In addition, we used an activity monitor to measure spontaneous behavior as a global predictor of pain and disability. Subsequently, we characterized biochemical changes that occur with CIA, assessing astrocyte and microglia hyperplasia and nerve damage. We validated the model for the study of analgesics by confirming the analgesic efficacy of the cyclooxygenase 2 (COX-2) inhibitor celecoxib. We finally assessed the influence of anti-TNF therapy on the nociceptive system in this model (and the methods of action), to compare it with the well-documented analgesic effect in humans with RA.

MATERIALS AND METHODS

Induction of arthritis. Adult male DBA/1 mice ages 10–12 weeks were used for all studies. Mice were housed in groups of 10 and maintained at a temperature of 21 ± 2°C (mean ± SEM) on a 12-hour light/dark cycle (7:00 AM to 7:00 PM), with food and water available ad libitum. All experimental procedures were approved by the UK Home Office and followed guidelines issued by the International Association for the Study of Pain. Mice were immunized by subcutaneous injection at the base of the tail with 2 50-μl injections of bovine type II collagen (2 mg/ml) in CFA (Becton Dickinson, Twickenham, UK), as described previously (3). Fourteen to 28 days following immunization, arthritis developed in the mice.

Assessment of arthritis. The thickness of each affected hind paw was measured daily with microcalipers (Kroeplin, Schüttern, Germany). Animals were scored for clinical signs of inflammation (3), as follows: 0 = normal, 1 = slight swelling and/or erythema, 2 = pronounced edematous swelling, and 3 = joint rigidity. Each limb was graded, thus allowing a maximum score of 12 per mouse. This experiment was repeated on 3 separate occasions.

Assessment of evoked pain (thermal and mechanical hyperalgesia). The mice were placed in the equipment used to assess hyperalgesia on at least 2 occasions prior to pain evaluation, in order to reduce stress-induced behavioral changes. Thermal hyperalgesia was assessed using the Hargreaves plantar apparatus (Ugo Basile, Varese, Italy), as described previously (9). Briefly, mice were placed in a Perspex box, and an increasing thermal stimulus was delivered to the plantar surface of the hind paw. The amount of time until lifting of the paw was recorded. The microprocessor-controlled Ugo Basile 37400 Plantar Von Frey apparatus was used to assess mechanically induced pain thresholds. Animals were placed in boxes of wire mesh, and an increasing force was applied to the hind paw at the rate of 3g/second. The force required to elicit lifting of the paw was taken as the withdrawal threshold. Allodynia was assessed before immunization (to establish a baseline level), prior to the onset of arthritis (14 days after immunization), and up to 28 days after the onset of arthritis, in the first hind paw to become arthritic. If both paws became arthritic on the same day, both hind paws were assessed and the average value was obtained. This experiment was repeated on 3 separate occasions.

Assessment of spontaneous behavior. The Laboratory Animal Behaviour Observation, Registration and Analysis System (LABORAS) is an automated system that detects vibrations evoked by movement of a single rodent in a cage. Pattern recognition software is then used to recognize and quantify behaviors, including grooming, mobility, climbing, immobility, and feeding (12,13). Animals were acclimatized to the equipment on 2 occasions prior to measurement. On the day of arthritis onset or 10 days after onset, naive and arthritic mice were placed in the LABORAS activity monitor (Metris, Hoofddorp, The Netherlands) overnight (18 hours). This experiment was repeated on 2 separate occasions.

COX-2 inhibition and anti-TNF therapy. To assess the influence of COX-2 on pain in CIA, animals were treated for 10 days, beginning from the day of onset, with 30 mg/kg celecoxib in 0.5% hypromellose by oral gavage, or with vehicle alone, twice daily. The paw diameter and clinical score were assessed daily, as described above. Thermal and mechanical hyperalgesia were assessed prior to the initiation of therapy, and 3, 7, and 10 days after the initiation of therapy, as detailed above.
To assess the influence of TNF on pain in CIA, animals were treated every other day with 100 μg soluble TNF receptor (STNFR)–Fc in phosphate buffered saline (PBS) intraperitoneally (14), beginning on the day of arthritis onset. Control animals received PBS only or an IgG2 anti-ragweed isotype control. Paw diameter, the clinical score, and thermal and mechanical hyperalgesia were assessed throughout, as detailed above. Because the responses in animals that received PBS and those in animals that received isotype control were comparable, PBS was used for further experiments. Animals were assessed in LABORAS for 6 hours, either immediately after administration of the first dose of anti-TNF or vehicle or 10 days after the initiation of therapy, to assess the influence of the drug on spontaneous behavior. This experiment was repeated on 2 separate occasions.

**Immunohistochemical analysis.** At various time points throughout the disease course, animals were killed by CO2 exposure, and the lumbar spinal cord and L4–L5 dorsal root ganglia (DRG) were excised, fixed in 10% formalin, and embedded in paraffin. Immunohistochemical analysis with a rabbit anti–glial fibrillary acidic protein (anti-GFAP) antibody (DakoCytomation, Glostrup, Denmark) was performed to detect astrocytes. Microglial activation in the spinal cord was assessed with a rat anti-mouse F4/80 monoclonal antibody (Serotec, Oxford, UK). Nerve damage was assessed using a rabbit anti–activating transcription factor 3 (anti–ATF-3) antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Antibody detection was performed using an avidin–biotin–peroxidase method (Vector, High Wycombe, UK) (15). The number of hyperplastic astrocytes (star-shaped glial cells) was quantified by 2 observers who were blinded to the treatment in 3 dorsal horn sections per spinal cord, and the mean number for each animal was determined. The number of ATF-3–positive neurons in the DRG was quantified in 3 sections per mouse by 2 blinded observers and expressed as a percentage of neuronal cell bodies.

**Statistical analysis.** Group mean values were analyzed by one-way analysis of variance, followed by the Dunnett’s multiple comparison test, when appropriate. Unpaired t-tests were used to assess the effect of therapy on astrocyte activation and nerve damage.

**RESULTS**

**Pronounced hyperalgesia during the course of CIA.** As reported previously, following the onset of arthritis, paw swelling and the clinical score increased significantly, reaching a maximum 7–10 days after the onset of CIA, and remained elevated throughout the 28-day study period (Figures 1A and B, respectively) (16). On the day of onset of clinical arthritis, significant reductions in both the mechanical (Figure 1C) and thermal (Figure 1D) pain thresholds were detected.
Maximum hyperalgesia to both mechanical and thermal stimuli was observed 7–10 days after the onset of arthritis. Mechanically induced pain thresholds increased 14 days after the onset of CIA but remained significantly reduced compared with those in naive mice, for up to 28 days after the onset of arthritis. In contrast, thermal thresholds returned to the levels observed in naive mice, 18–24 days after arthritis onset.

Analysis of changes in spontaneous behavior during CIA. We compared spontaneous behavior in arthritic and nonarthritic mice using the LABORAS activity monitor. Animals were assessed overnight, beginning at 4:00 pm and ending 18 hours later, because this is when mice are most active. Significant reductions in the amount of time spent climbing and in locomotion were observed in arthritic mice at the onset of disease and 10 days after onset of CIA (Figure 2A). In addition, the amount of time spent immobile was increased, and the amount of grooming time decreased in mice with arthritis (Figure 2B). An overall reduction in distance traveled was observed in mice with arthritis, while the speed of movement, eating, and drinking was not affected (data not shown).

Astrocyte activation in the dorsal horn in CIA, in the absence of microglial activation. We next assessed changes in the nociceptive system that are known to occur in chronic pain states (17). Astrogliosis was observed in the lumbar spinal cords of mice with CIA, as detected by GFAP labeling (Figure 3A). The number of hyperplastic astrocytes (i.e., the number of GFAP-labeled star-like cells) increased significantly beginning 10 days after the onset of arthritis (Figure 3C). At no time during the first 28 days after the onset of arthritis were activated microglia detected in the spinal cords of arthritic mice, as assessed by F4/80 staining (data not shown). This is in contrast to models of neuropathy, in
Figure 4. Effect of cyclooxygenase 2 inhibition on pain and inflammation in collagen-induced arthritis. Celecoxib or vehicle was administered twice daily for 10 days, beginning on the day of arthritis onset. Paw diameter (A), clinical score (B), mechanical hyperalgesia (C), and thermal hyperalgesia (D) were assessed prior to therapy (day 0) and throughout the 10-day therapy period. Values are the mean ± SEM results from 8 mice per group and are representative of 2 experiments. g = grams force; s = seconds. * = P < 0.05; ** = P < 0.01; *** = P < 0.001.

Figure 5. Effect of anti–tumor necrosis factor (anti-TNF) therapy on pain and inflammation in collagen-induced arthritis. Anti-TNF (soluble TNF receptor [sTNFR]-Fc), IgG isotype control, or phosphate buffered saline (PBS) was administered on alternate days for 10 days, beginning on the day of arthritis onset. Paw diameter (A), clinical score (B), mechanical hyperalgesia (C), and thermal hyperalgesia (D) were assessed prior to therapy (day 0) and throughout the 10-day therapy period. Values are the mean ± SEM results from 8 mice per group and are representative of 2 experiments. g = grams force; s = seconds. * = P < 0.05; ** = P < 0.01; *** = P < 0.001, versus vehicle.
which microglial activation is marked (18). Because astrocyte activation is associated with neuropathic conditions, we addressed the question of whether nerve damage was also a feature of CIA, by assessing ATF-3 expression in the L4–L5 DRGs (Figures 3B and D). A small but statistically significant increase in the percentage of ATF-3-positive cells was observed in the DRGs of arthritic mice, beginning 14 days after the onset of arthritis. This suggests that there is a small yet significant increase in the level of nerve damage in CIA.

**Effect of COX-2 inhibition on inflammation and pain in CIA.** To validate CIA as a model for the study of pain and analgesics, we assessed the action of the COX-2 inhibitor celecoxib on pain in this model. Because the most pronounced evoked pain was detected 10 days after arthritis onset (Figure 1), we assessed therapy for 10 days following the onset of arthritis. Celecoxib significantly reduced the clinical score by day 3 after the onset of CIA (Figure 4B). A reduction in paw swelling was also observed, although this did not reach statistical significance (Figure 4A). As predicted, celecoxib significantly reduced mechanical hyperalgesia at day 10 after the initiation of therapy (Figure 4C) and thermal hyperalgesia (Figure 4D) beginning on day 7 after the initiation of therapy.

**Effect of anti-TNF therapy on inflammation and pain in CIA.** We next assessed whether anti-TNF therapy (sTNFR-Fc) was effective against pain. As previously reported, anti-TNF therapy significantly reduced swelling and the clinical score in CIA (Figures 5A and B). In addition, a significant reduction in mechanical hyperalgesia was observed beginning 7 days after the onset of arthritis, and a significant reduction in thermal hyperalgesia was observed beginning 10 days after the onset of arthritis.

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**Figure 6.** Effect of anti–tumor necrosis factor (anti-TNF) therapy on behavior and astrocyte activation in collagen-induced arthritis. A and B, Animals were treated with a soluble TNF receptor II (sTNFRII) decoy or phosphate buffered saline (PBS). Immediately after the first injection (day of onset) (A) or after the final injection (8 days after onset) (B), animals were placed in the Laboratory Animal Behaviour Observation, Registration and Analysis System activity monitor, and their behavior was assessed for 18 hours. Age-matched naive control mice were also studied (n = 6–8 per group). The amount of time spent climbing, mobile, immobile, and grooming was quantified. C and D, After 10 days of treatment with anti-TNF or PBS, spinal cords and L4–L5 dorsal root ganglia (DRG) were obtained and fixed in 10% formalin and embedded in paraffin. Immunohistochemical analysis for glial fibrillary acidic protein was performed on the lumbar spinal cord, and the number of hyperplasic astrocytes in the spinal cord was quantified (C). Immunohistochemical analysis for anti–activating transcription factor 3 (ATF-3) was performed on the L4–L5 DRG, and the number of ATF-3-positive neurons was counted and expressed as a percentage of total cell bodies (D). Values are the mean and SEM and are representative of 2 experiments. NS = not significant. * = P < 0.05; ** = P < 0.01.
hyperalgesia was detected at all time points assessed (Figure 5C and D, respectively).

**Effect of anti-TNF therapy on spontaneous behavior in arthritic mice.** In order to assess whether anti-TNF therapy changed spontaneous behavior, we placed mice in the LABORAS activity monitor for 6 hours, after administration of the first dose of anti-TNF therapy or after 10 days of therapy. Following administration of the first dose of anti-TNF therapy, the amount of time that mice spent climbing was significantly increased, although no change in locomotion or immobility was detected (Figure 6A). Similarly, after 10 days of anti-TNF therapy, the amount of time spent climbing was significantly increased, to the level observed in naive mice (Figure 6B). These measures provide a quantitative approach to behavioral changes resembling symptoms in humans.

**Effect of anti-TNF therapy on astrocyte activation in the spinal cord.** Finally, we used GFAP staining to assess whether anti-TNF therapy altered astrocyte activation in the spinal cord, and assessed nerve damage via ATF-3 expression in the DRGs supplying the joint, 10 days after the onset of arthritis. Anti-TNF therapy reduced the number of hyperplasic astrocytes in the spinal cord by 50% \( P < 0.05 \) (Figure 6C). In contrast, no significant difference in ATF-3 expression was observed following anti-TNF therapy (Figure 6D).

**DISCUSSION**

This is the first report to describe the use of standard behavioral techniques, which are used regularly in models of monarthritis and neuropathy (9,19,20), to study hyperalgesia in a model of polyarthritis. Until \( \sim 10 \) days after the onset of arthritis, a good correlation between swelling (reflecting inflammation) and hyperalgesia was observed. Both thermal and mechanical hyperalgesia decreased at later stages of the disease. Expression of the inflammatory cytokines TNF\( \alpha \) and interleukin-1\( \beta \) (IL-1\( \beta \)) has been shown to increase in the joints of mice with CIA, beginning at the time of onset of arthritis, with a subsequent decrease 10 days after onset (21,22). It is possible that the lack of algesiogenic (pain-inducing) mediators in the joint in later stages of CIA results in decreased hyperalgesia. Alternatively, as the disease progresses, the level of disability increases, which may result in an apparent reduction in hyperalgesia. We concluded from the time course study that it would be useful to assess analgesics for up to 10 days after the onset of arthritis, when hyperalgesia was most reliably detected.

The activity of rodents has been used previously as a surrogate for evoked pain measurements in CFA-induced monarthritis (23). Here, we used an automated activity monitor (LABORAS) that quantifies many spontaneous behaviors, including climbing and grooming (13). Arthritis resulted in less time spent mobile, as reported for CFA-induced arthritis (23). In addition, a large reduction in the amount of time spent climbing and an increase in immobility were observed with arthritis. Less predictably, a significant reduction in the amount of time spent grooming was detected in arthritic animals. A lack of grooming is thought to represent sickness behavior, because peripheral IL-1\( \beta \) administration to mice reduced the amount of grooming behavior (24). Because CIA is a systemic disease, an increase in cytokine levels and cachexia is likely. This is analogous to RA, in which malaise and fatigue are prominent features.

Astrocyte and microglia activation are known to be critical components of neuropathic conditions, and down-regulation of astrocyte/microglia activity is analgesic in these conditions (17). Astrocyte activation in arthritis has been reported but is not well characterized (25,26). We showed a large increase in the number of hyperplastic astrocytes in the lumbar spinal cord, beginning 10 days after the onset of arthritis, with no change in microglia number or hyperplasia for up to 28 days after onset. In adjuvant-induced arthritis in rats, both astrocyte hyperplasia and microglia hyperplasia have been shown. This indicates that different inflammatory conditions may induce different cellular changes in the nervous system.

Because astrocyte activation is strongly associated with neuropathy (27), we assessed whether peripheral nerve damage occurs in CIA, using ATF-3 as a marker of damage. A small but significant increase in the level of ATF-3 was observed in the DRGs supplying the ankle, reaching \( \sim 2\% \) of the L4–L5 DRG 14–28 days after the onset of arthritis. Because only a very small percentage of the nerves from the L4–L5 DRG innervate the joint, this could indicate significant damage of neurons supplying the arthritic joint. This would need to be addressed further using retrograde labeling of nerves coupled with ATF-3 staining. A recent study showed that infection of DRG neurons in culture with herpes simplex virus—expressing ATF-3 results in increased neuronal outgrowth (28). An alteration in the innervation of arthritic joints has been shown in RA, indicating potential nerve damage and/or outgrowth (29). However, because GFAP expression precedes ATF-3 expression, it is unlikely that astrocyte activation is occurring as a result of nerve damage.
In order to validate the model for the study of analgesics, we assessed the influence of a COX-2 inhibitor, celecoxib, as a positive control. As observed with other models, celecoxib was effective against both mechanical and thermal hyperalgesia (30–32). We then assessed the influence of TNF in CIA. As in models of acute inflammation (8,9), we showed that anti-TNF had profound analgesic properties in CIA. Therapy also partially normalized the spontaneous behavior abnormalities detected with LABORAS. Anti-TNF therapy decreased the number of activated astrocytes in the spinal cord. This is the first study to show that anti-TNF reduces astrocyte activity and may help account for its analgesic actions in models of neuropathy (33).

We have not addressed the site of anti-TNF action. It is known that various cells in the arthritic joint secrete TNFα, especially macrophages (34), and we have shown that inflammation induces TNF receptor I (TNFRI) expression on DRG neurons (9). In other models of arthritis, leakiness in the blood–brain barrier has been observed (35,36). If leakiness occurs in CIA, blockade of TNF by sTNFR-Fc could act in the spinal cord by inhibiting astrocyte activity, because these cells have been shown to express TNFR when activated (37,38). Although anti-TNF therapy is known to be neuroprotective (39), ATF-3 expression was not affected by anti-TNF, indicating a distinct action in reducing astrocyte activity.

In conclusion, we have shown that CIA in the DBA/1 mouse (the best-documented animal model of human RA) can be used to assess pain and analgesics, and have demonstrated that astrogliosis occurs in CIA in the absence of microglial activation and with a significant yet small neuropathic component. Finally, we have demonstrated that anti-TNF therapy is analgesic in this model, with efficacy comparable with that of COX-2 inhibition, and reduces astrocyte activation in the spinal cord. The CIA model is thus suitable for testing not only antiinflammatory but also analgesic drugs for potential use in RA.

AUTHOR CONTRIBUTIONS

Dr. Inglis had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Inglis, Anand, Williams.

Acquisition of data. Inglis, Notley, Essex.


Statistical analysis. Inglis.

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