Temporal Changes in Inflammatory Mediator Concentrations in an Adjuvant Model of Temporomandibular Joint Inflammation

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Aims: *To determine temporal changes in the concentrations found in the temporomandibular joint (TMJ) and trigeminal ganglion of 3 specific classes of inflammatory mediators commonly linked with conditions of joint inflammation. The intent was to determine whether concentrations of the neuropeptide calcitonin gene–related peptide (CGRP), the neurotrophin nerve growth factor (NGF), and the proinflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) are altered in the trigeminal ganglion and TMJ tissues during various stages of adjuvant-induced inflammation of the rat TMJ.* ***Methods:*** *Adult male rats received bilateral TMJ injection of complete Freund's adjuvant (CFA), while control rats did not receive CFA treatment. The trigeminal ganglion and TMJ tissues were collected at 2 days, and 2, 4, and 6 weeks postinjection and analyzed using either radioimmunoassay or enzyme-linked immunosorbent assay.* ***Results:*** *In the trigeminal ganglion, both CGRP and NGF concentrations were significantly elevated in comparison to controls from 2 days to 4 weeks; however, the patterns of increase differed. Concentrations of each inflammatory mediator were significantly elevated in the TMJ tissues of CFA-injected animals at 2 days and continued to be significantly elevated throughout the 6-week period. CGRP content remained at peak levels from 2 days through 6 weeks, while peak content for NGF, IL-1β, and TNF-α was found at 2 days through 2 weeks.* ***Conclusion:*** *The results suggest that the development of CFA-induced inflammation of the TMJ was accompanied by a variable increase in the concentration of different classes of inflammatory mediators in both the trigeminal ganglion and TMJ tissues, which implies that each class of inflammatory mediator may play a significant role during different stages in the onset and exacerbation of the inflammatory process.* J OROFAC PAIN 2005;19:34–40

Key words: calcitonin gene-related peptide, inflammation, interleukin-1β, nerve growth factor, temporomandibular joint, trigeminal ganglion, tumor necrosis factor-α

Joint inflammation often results in increased concentrations of many different classes of inflammatory mediators. Neuropeptides, neurotrophins, and cytokines are altered in both acute and chronic joint inflammation, contributing to the onset and exacerbation of symptoms.[1,2] However, there are few data to indicate how these mediators are affected during the progression from acute to chronic inflammation. Although each inflammatory mediator potentially plays an important role in the development and maintenance of inflammation, their specific temporal contributions to the overall inflammatory process are still in question. Understanding this process will facilitate the selection of appropriate treatment during each inflammatory stage.

The neuropeptide calcitonin gene–related peptide (CGRP) plays a significant role in joint inflammation and subsequent cartilage and bone destruction.[3] CGRP is synthesized in the cell bodies of nociceptive neurons and is typically transported to peripheral nerve endings of Aδ and C-fibers. Following stimulation of these peripheral terminals, release of CGRP has been shown to produce vasodilatation and plasma extravasation.[4] Additionally, studies have shown that the release of various inflammatory mediators within inflamed tissues can induce CGRP release from nociceptive nerve endings.[5,6] Fibers showing CGRP immunoreactivity are present within the synovium of different joints,[7–10] including the temporomandibular joint (TMJ).[11–14] Also, CGRP is involved in the perpetuation and exacerbation of arthritis-related inflammations, and depletion of CGRP in diseased joints is correlated with decreased levels of other inflammatory mediators.[15,16]

The neurotrophin nerve growth factor (NGF) is produced in peripheral tissues, taken up by nerve terminals, and retrogradely transported to the neuronal cell bodies.[17] One of NGF's normal physiologic actions is thought to be the maintenance of neuropeptide content within nerve cell bodies,[18,19] particularly those of sensory neurons involved in nociception.[20,21] Those studies have shown that blockage of retrograde axoplasmic transport of NGF with capsaicin application[20,21] or antagonists[22] results in a decreased concentration of CGRP in dorsal root ganglia, which indicates that NGF may be involved in regulating the synthesis and release of CGRP. Additionally, increased concentrations of NGF have been demonstrated in an adjuvant-induced model of arthritis and are thought to contribute to the generation of neurogenic inflammation and its associated effects.[23,24] A similar situation may occur in the trigeminal ganglion during TMJ inflammation. Thus, the regulatory effect of NGF on neuropeptide expression may be of particular importance during TMJ inflammation.

The proinflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) have also been studied extensively in joint inflammation,[25–27] particularly in regard to their involvement in inflammation of the TMJ.[28,29] Abnormal cytokine production or receptor expression has been related to the induction and persistence of inflammation in arthritic joints. Further, IL-1β and TNF-α are important because they initiate several critical events in the acute and chronic stages of inflammation and tissue damage during rheumatoid arthritis, including increased production of additional cytokines and other proinflammatory mediators.[30]

Antigen-induced arthritis is a documented model for the study of rheumatoid arthritis.[31–33] Numerous investigators have examined these inflammatory mediators within joint tissues to determine their effects within the dorsal root ganglia.[1,34,35] While limb joints remain the primary focus of studies on joint inflammation, a growing body of literature has documented the distribution of and morphological changes associated with the presence of different inflammatory mediators in the TMJ.[11–14,27–29]

Many TMJ disorders contain an inflammatory component, but their mechanisms and pathophysiology have not been clearly delineated. Investigators have examined the concentration of various inflammatory mediators in the TMJ synovial fluid of humans[15,16] and after adjuvant administration to different animal models.[13,28,29,33,36–38] However, the animal studies have typically focused upon relatively short time periods after adjuvant administration to the TMJ and have not analyzed the 3 different classes of inflammatory mediators (CRGP, NGF, and cytokines IL-1β and TNF-α) in both the acute and chronic inflammatory stages. Therefore, the aim of this investigation was how concentrations of these 3 specific classes of inflammatory mediators commonly linked with conditions of joint inflammation change over time.

Materials and Methods

Adjuvant Injection

Forty adult male Sprague-Dawley rats (200 to 250 g) were anesthetized with an intramuscular injection of a ketamine (90 mg/kg) and Rompun (8.7 mg/kg; Bayer). Each animal received a bilateral injection in the TMJ with 50 μg complete Freund's adjuvant (CFA) (*Mycobacterium tuberculosis;* Sigma) suspended in 50 μL paraffin oil. Verification of the TMJ injection site and evidence of an inflammatory response have been previously reported.[37,38] Forty additional animals were used as age-matched, uninjected controls. Vehicle controls were not included in this study, as it has been shown that a contralateral crossover effect, whereby the vehicle itself affects levels of inflammatory mediators in both the peripheral and central nervous systems on the opposite side from the injection, can occur in response to the vehicle.[37,39] The CFA-treated animals were monitored for any postinjection trauma, and their weights were measured daily. None of the animals in this study experienced symptoms of trauma (eg, respiratory

distress, inability to chew, immobilization), and no data were excluded or altered because of this issue. Animals typically lose a small percentage of their body weight for the first 2 to 3 days postinjection and then return to normal weights, as previously described.[38,40] Food and water were provided ad libitum, and all animals were maintained under normal 12 h light:12 h dark cycles at 23°C.

Tissue Preparation

Both the CFA-treated and control animals were randomly divided into 4 groups of 20 animals each (10 injected and 10 control) and sacrificed via an overdose injection of Nembutal (Abbott Laboratories) (100 mg/kg intraperitoneally [IP]) at various time intervals to mimic conditions of acute (2 days), intermediate (2 weeks), and chronic (4 and 6 weeks) inflammation. The trigeminal ganglion and TMJ tissues (synoviums, retrodiscal tissues, and articular discs) were extirpated bilaterally, individually frozen in liquid nitrogen, and homogenized with 2 mol/L acetic acid in 4% ethylenediaminetetraacetic acid (EDTA).[10] After the samples were centrifuged, the supernatants were set aside while the pellet was again washed with the 2 mol/L acetic acid and 4% EDTA. This was then centrifuged, and the 2 supernatants were combined and lyophilized. Later, they were rehydrated and prepared for either enzyme-linked immunosorbent assay (ELISA, for NGF in trigeminal ganglion and TMJ, IL-1β in TMJ, and TNF-α in TMJ) or radioimmunoassay (RIA, for CGRP in the trigeminal ganglion and TMJ). Each assay group consisted of 10 individual samples from both CFA-injected and control animals at each time period. All procedures and sacrifice protocols were approved by the Baylor College of Dentistry Institutional Animal Care and Use Committee.

ELISA for NGF, IL-1β, and TNF-α

Quantitative measurement of NGF, IL-1β, and TNF-α was performed in duplicate using commercially available sandwich ELISA kits (Promega for NGF and R&D Systems for IL-1β and TNF-α). Flat-bottom 96-well plates precoated with either IL-1β, TNF-α, or NGF antibodies were used, and samples were run in duplicate along with standards for each assay. The captured NGF, IL-1β, or TNF-α was bound by a second specific monoclonal antibody, and nonspecific binding was blocked with bovine serum albumin. After washing, the bound antibody was reacted with an antibody conjugated to horseradish peroxidase. Following incubation with a chromogenic substrate, the color change was measured using a Molecular Devices SpectraMax 250 ELISA Plate Reader. A standard Folin Lowry protein assay was performed to normalize the protein content between all samples. Results were then expressed as pmol NGF-ir, IL-1β-ir, or TNF-α-ir (*ir* stands for immunoreactivity).

RIA for CGRP

Duplicate aliquots of the rehydrated supernatants were assayed using RIA kits (Phoenix Pharmaceutical) specific for CGRP according to directions provided by the manufacturer. Briefly, supernatant from each sample and standards were added to polystyrene test tubes followed by the addition of primary antibody (rabbit anti-rat CGRP) and incubation overnight at 4°C. Iodinated anti-CGRP peptide was then added and incubated overnight at 4°C. Secondary antibody (goat anti-rabbit IgG) was subsequently added in combination with normal rabbit serum and RIA buffer in a typical competitive assay method and incubated at room temperature for 90 minutes. The samples were then centrifuged at 3,000 rpm at 4°C for 20 minutes, and the contents in the resulting pellet were calculated using a Cobra Auto Gamma radiation counter (Packard Instruments). Each sample was normalized for protein content to make comparisons between samples and compared against a standard curve.

Statistical Analysis

Duplicate samples for each tissue were averaged, and the samples (n = 10 for each time period) were compared. Differences in CGRP, NGF, IL-1β, and TNF-α content between CFA-injected and control animals were evaluated using 1- or 2-way ANOVA, where appropriate. Findings were considered statistically significant where $P \leq .05$.

Results

Trigeminal Ganglia

The CGRP concentrations in the trigeminal ganglia of CFA-injected animals were significantly elevated in comparison to control animals at 2 days, 2 weeks, and 4 weeks, but not at 6 weeks (Fig 1). The NGF concentration was significantly elevated in the trigeminal ganglia of CFA-injected animals in comparison to control animals at 2 days, 2 weeks, and 4 weeks, but not at 6 weeks (Fig 2). In

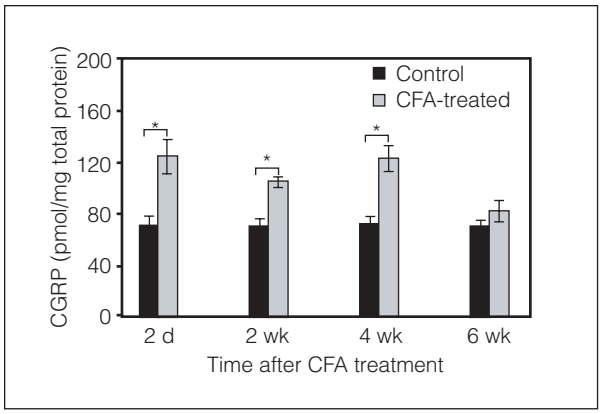

Fig 1 Trigeminal ganglion CGRP-ir concentrations. Trigeminal ganglia were removed and analyzed by RIA for changes in CGRP content. Concentrations of CGRP were significantly increased in comparison to controls at all time periods except 6 weeks. $*P \leq .05$. In all figures bars indicate standard deviation.

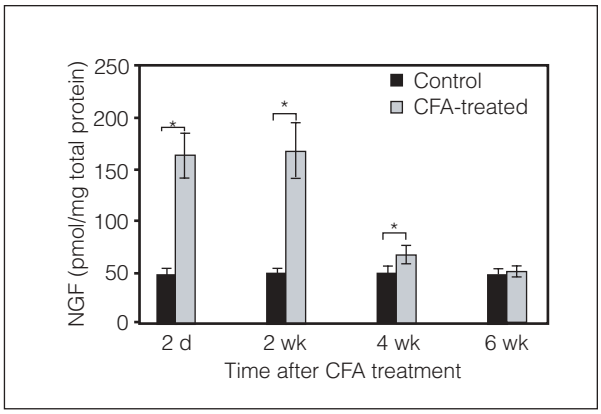

Fig 2 Trigeminal ganglion NGF-ir concentrations. Trigeminal ganglia were removed and analyzed by ELISA for changes in NGF content. Concentrations of NGF were significantly increased in comparison to controls at all time periods except 6 weeks. $*P \leq .05$.

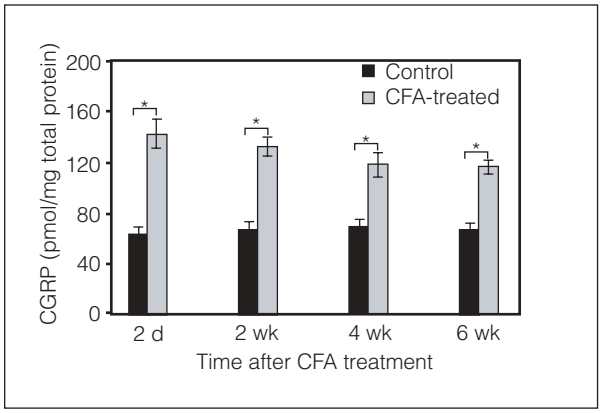

Fig 3 TMJ CGRP-ir concentrations. TMJ tissues were removed and analyzed by RIA for changes in CGRP content. No significant differences in CGRP concentration were found between any of the time periods. Concentrations of CGRP were significantly increased in comparison to controls at all time periods. $*P \leq .05$.

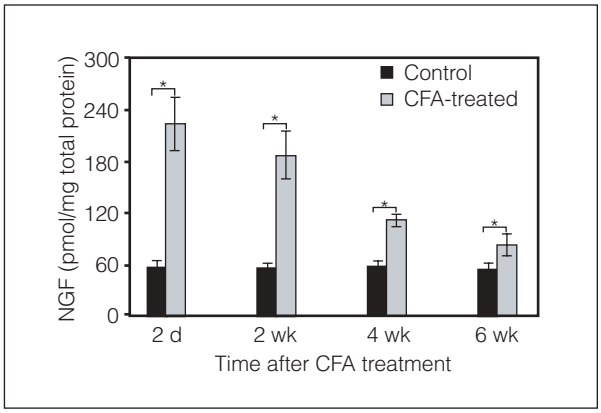

Fig 4 TMJ NGF-ir concentrations. TMJ tissues were removed and analyzed by ELISA for changes in NGF content. Concentrations of NGF were significantly increased in comparison to controls at all time periods except 6 weeks. There was a significant difference between concentrations at 4 and 6 weeks and those measured at 2 days and 2 weeks. $*P \leq .05$.

contrast to CGRP, NGF concentrations at 4 weeks were significantly reduced in comparison to concentrations at 2 days or 2 weeks.

TMJ Tissues

The pattern of inflammatory mediator expression observed in the TMJ tissues was different from that seen in the trigeminal ganglion. The CGRP concentration, which remained relatively constant over the 6-week period, was significantly elevated in comparison to controls at all time periods investigated (Fig 3). On the other hand, NGF concentrations followed a trend similar to that observed in the trigeminal ganglion. The concentration of NGF in the TMJ tissues peaked at 2 days postinjection and then declined steadily over time (Fig 4).

The IL-1β and TNF-α concentrations in the inflamed TMJ tissues generally demonstrated a pattern similar to that of NGF. The peak IL-1β and TNF-α concentrations were seen at 2 days, and lower values were observed at later time periods (Figs 5 and 6).

Discussion

Understanding the underlying mechanisms of the inflammatory process has led to an increased interest in the role that neuropeptides, neurotrophins,

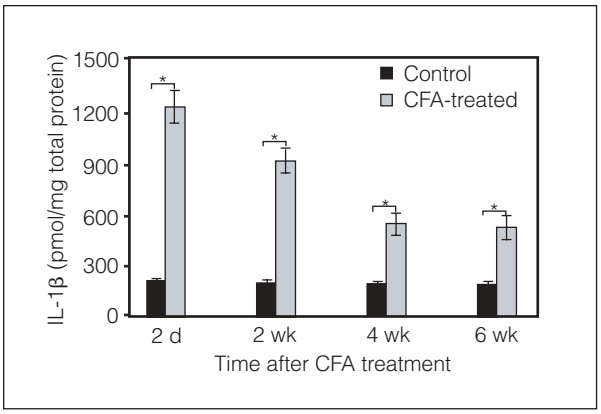

Fig 5 TMJ IL-1β-ir concentrations. TMJ tissues were removed and analyzed by ELISA for changes in IL-1β conent. Concentrations of IL-1β were significantly increased in comparison to controls at all time periods. Concentrations of IL-1β were at their peak at 2 days after CFA treatment and were significantly lower at both 2 and 4 weeks. Concentrations at 6 weeks were not significantly different from 4 weeks. $*P \leq .05$.

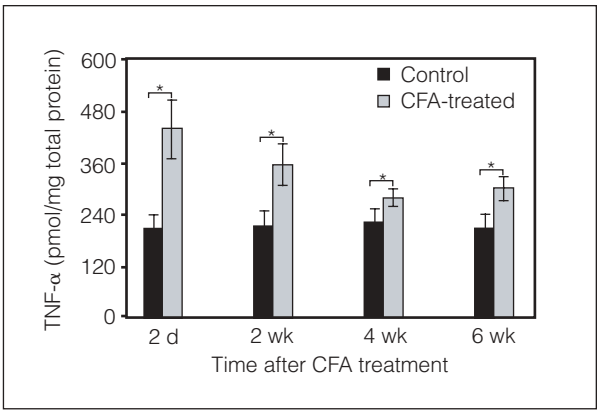

Fig 6 TNF-α-ir concentrations. TMJ tissues were removed and analyzed by ELISA for changes in TNF-α content. Concentrations of TFN-α were significantly increased in comparison to controls at all time periods. Concentrations of TNF-α were at their peak at 2 days after CFA treatment. Values were not significantly different between any time point and the time period proceeding it, but concentrations at 4 and 6 weeks were significantly different from those at 2 days. $*P \leq .05$.

and cytokines play in inducing or exacerbating the symptoms associated with joint inflammation and destruction. A description of the temporal changes that occur during the different stages of inflammation is critical for the determination of the best methods for the treatment of patients presenting with different manifestations of inflammatory joint disorders. While most studies have used limb joints as a model to focus on this process, little is known about the progression of events that takes place during inflammation of the TMJ. Although reports indicate an inflamed TMJ contains different neuropeptides, neurotrophins, and cytokines, most studies have focused on the concentration of these mediators during specific, brief time periods.[11–14,27–29] Each inflammatory mediator plays an important role in the initiation and progression of inflammation; however, the role that each mediator plays has not been determined. Thus, the aim of this study was to determine temporal changes in the concentration of 3 different classes of inflammatory mediators commonly thought to participate in joint inflammation during various stages of TMJ inflammation.

Using a model of adjuvant-induced inflammation of the TMJ, the authors previously showed that observable histologic changes within the soft tissues of the joint occurred during the acute period of inflammation and led to a propagation over time of this inflammatory response.[37,38] While histologic evidence of inflammation exists, no behavioral changes during later stages of inflammation were previously observed.[40] These findings are consistent with those seen in other studies of the TMJ.[13,28,29] However, the current study has demonstrated that the concentrations of various inflammatory mediators in the periphery may change over time and has revealed unexpected relationships.

Specifically, the results of the present study indicate that concentrations of proinflammatory cytokines and neurogenic-related peptides remained significantly elevated in the TMJ tissues of the CFA-injected animals in comparison to the uninjected controls at all time periods investigated. However, in the trigeminal ganglion, concentrations of CGRP and NGF in the CFA-injected animals had returned to control levels by 6 weeks. Previous studies have suggested that NGF is necessary to maintain normal concentrations of CGRP in sensory neurons[18,20]; however, the present data suggest this may only apply during the first 4 weeks of an adjuvant-induced inflammation. During the first 4 weeks, the present data support the hypothesis that NGF is necessary both to maintain the normal production of CGRP and to induce the increased production of CGRP in the trigeminal ganglion. The present data also showed an apparent lag between the initial decrease in trigeminal ganglion NGF levels and CGRP levels, as reported elsewhere.[18,20]

At the later time period, there appeared to be a difference between peripheral and ganglionic NGF and CGRP concentrations. NGF and CGRP concentrations within the trigeminal ganglion were at

control levels 6 weeks after CFA-induced inflammation within the TMJ, but both peptide concentrations were significantly greater in the TMJ tissues. The assayed concentrations of CGRP in the TMJ tissues probably do not reflect much of the released neuropeptide, since CGRP is degraded quickly following release, but would suggest that CGRP is being stored at greater concentrations in the periphery. The greater concentrations of peripheral CGRP in the TMJ tissues at 6 weeks may also reflect the axonal sprouting that has been described following an inflammation.[41] The lack of CGRP translation above control levels at 6 weeks would further support the idea that greater storage occurs in the periphery.

Additionally, at 6 weeks, NGF concentrations were also greater in the TMJ tissues, but the trigeminal ganglion concentrations were no different than controls, implying that the peripheral NGF is either not taken up at the nerve terminals and/or not transported back to the nerve cell bodies. NGF signals travel via 2 distinct receptors: the low-affinity nonspecific p75 receptor that binds all neurotrophins, and the high-affinity NGF-specific receptor Trk A. The authors' results indicate that there may be fewer Trk A or p75 receptors expressed in the inflamed TMJ. Thus, NGF by itself may not be necessary to support a persistent neurogenic inflammation.

An understanding of the levels of the cytokines may also offer another possible insight into some of the events supporting a peripheral chronic inflammatory process. Although TNF-α and IL-1β both peaked early, and there are several studies to suggest that they are important inflammatory mediators during the acute inflammation, the 6-week data presented here suggest that they maintain significant levels in the TMJ tissues. Although concentrations of the proinflammatory cytokines decreased considerably by 6 weeks, they may act in concert with NGF and underlie the maintenance phase of a persistent inflammation. In the current study, it was observed that levels of the inflammatory mediators remained elevated even 6 weeks after CFA injection, which indicates that this model can produce a chronic TMJ inflammation and could be used to investigate potential treatments.

Adjuvant-induced inflammation as an arthritic model has been used extensively for studying changes that take place in joint tissues. Limb studies indicate that CGRP,[1,10,13,33] NGF,[22,24] and IL-1β or TNF-α[26,27] concentrations are altered by the injection of adjuvant and may play a role in symptoms observed during inflammation of joint tissues. However, these studies have typically investigated changes that occur during relatively short time periods and have not looked at changes that occur as the inflammatory response progresses. This study provides valuable baseline information on various classes of inflammatory mediators during the progression of TMJ inflammation. Further studies will elucidate how these factors interact with one another to exacerbate symptoms associated with TMJ inflammation.

Acknowledgments

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