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Trigeminal nerve stimulation triggers oral mast cell activation and vascular permeability

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ABSTRACT

Background: The nervous system contributes to the pathophysiology of allergic and inflammatory diseases, including oral inflammation. Mast cells (MCs) are involved in their pathogenesis through proinflammatory mediator release.

Objective: To investigate the effect of trigeminal nerve (TN) stimulation compared with sham operation on MC activation and oral vascular permeability in the gingiva, palate, buccal mucosa, and tongue of the rat and to examine the possible role of substance P using rats treated with capsaicin as neonates to deplete substance P.

Methods: Six male Sprague-Dawley rats (250 g) were anesthetized and injected intravenously with Evans Blue (EB). Six other rats were injected neonatally with capsaicin (n = 3) or solvent (n = 3) and then injected with EB when they reached 250 g. The mandibular branch of the TN was stimulated for 1 minute (n = 3), and the remaining rats (n = 3) were subjected to sham operation. The ipsilateral and contralateral sides of the mouth were examined for EB extravasation, and tissue sections were removed for light and electron microscopy.

Results: TN stimulation resulted in EB extravasation in the ipsilateral side compared with the contralateral side or the ipsilateral side of sham-operated rats. Significant degranulation of MCs also was evident only on the ipsilateral side ($P < .0001$). There was no difference in MC degranulation between the vehicle- and capsaicin-treated rats, implying that neuropeptides other than substance P may be involved.

Conclusion: This is the first time that TN stimulation has been shown to result in MC activation and oral vascular permeability, suggesting that MC inhibitors may be used for the treatment of oral inflammatory diseases.

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Introduction

Mast cells (MCs) are critical for allergic diseases, innate and acquired immunity, and inflammation through the release of numerous mediators.[1–4] These are often released selectively without degranulation[5] and have potent vasodilatory, inflammatory, and nociceptive properties.[3] MCs secrete preformed and newly generated tumor necrosis factor (TNF)[6] and adenosine triphosphate, bradykinin, platelet-activating factor, prostaglandins, serotonin, and tryptase.[7] These can excite sensory nerve fibers, thus resulting in a feedforward loop and potentiation of neurogenic inflammation.[8] MCs also stimulate T cells through TNF and cell-surface costimulatory molecules.[9,10]

Mast cells participate in the brain–skin axis[8] as targets of corticotropin-releasing hormone (CRH) and related peptides.[11] In fact, MC-dependent skin inflammation is impaired in the absence of sensory nerves.[12] The authors previously showed that trigeminal nerve (TN) activation stimulates dural MCs, thus contributing to pathogenesis of migraines.[13,14] Moreover, MCs augment adaptive immunity by recruiting dendritic cells to infected tissues.[15] MCs are considered critical in allergy and innate immunity in the skin[16] and might promote and regulate inflammation.[17]

Electrical stimulation of peripheral nerves increases vascular permeability and plasma extravasation in the oral cavity.[18,19] The role of MCs in such changes has not been investigated or has been insufficiently clarified. The aim of the present study was to

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investigate the effect of TN stimulation (TNS) on MC activation and vascular permeability in the oral cavity of rats and the possible involvement of substance P (SP) because it has been localized in the TN.[20,21]

Methods

Male Sprague-Dawley rats (N = 12; Charles River Laboratories, Cambridge, Massachusetts) were kept from birth until they were used (at 250 g) under standard living and feeding storage conditions at a 10-hour light and 14-hour dark cycle and were provided with food and water ad libitum. Some rats (n = 3) were injected with capsaicin, whereas others (n = 3) were treated with vehicle neonatally. The remaining rats (n = 6) were not treated neonatally and were kept on their cages until they were used (at 250 g). Of these rats, 3 were subjected to sham operation and the remaining 3 underwent TNS, as described below.

Evans Blue Extravasation

Rats were anesthetized with a combination of 0.5 mL of xylazine HCl and 0.5 mL of ketamine HCl (20 mg/mL each) intraperitoneally. After the animal was asleep, 0.6 mL of 1% Evans Blue (EB) in normal saline was injected into the tail vein. EB binds to large-molecular-weight proteins (eg, albumin) in the blood and serves as an indicator of vascular permeability.[22]

Neonatal Capsaicin Pretreatment

Capsaicin treatment was as follows.[23] Littermates (n = 3) were injected subcutaneously in the first 2 days of life with 50 mg/kg of capsaicin (Polysciences, Inc, Warrington, Pennsylvania) diluted in 0.05 mL of a solution containing 0.9% NaCl, 100% ethanol, and Tween 80 (8:1:1) or a solvent (n = 3, control). During capsaicin injection, animals were kept in a tent containing an aerosol of isoproterenol (0.25 mg/mL for 10 minutes). After injection, all neonates were returned to their cages. They were maintained on a diurnal lighting cycle and allowed access to food and water ad libitum. Eight weeks later, TNS was carried out as described below. The 6 rats were sacrificed over carbon dioxide and decapitated immediately after TNS. The oral cavity was exposed and photographed. EB staining appeared dark blue owing to the vascular background. Tissue specimens from the tongue, palate, buccal mucosa, and gingiva were taken and prepared for morphologic examination of MCs.

Surgical Exposure of the TN

The mandibular branch of the TN on the right side was exposed surgically as it branches off (Fig 1A). During the surgical exposure of the nerve, anesthesia was maintained by inhalation of methoxyflurane. A vertical incision was made distal to the ascending ramus of the mandible and in front of the ear on the right side. A second incision was made from the top of the first incision in an oblique direction toward the eye of the rat. The mandible was gently moved forward and to the outside to expose the TN as it exits from the brain (Fig 1B). This protocol was approved by the Tufts Medical Center animal care and use committee.

Stimulation of the TN

In some rats (n = 3), the mandibular branch of the TN was stimulated using a disposable nerve stimulator (VARI-STEM, III, Bristol-MyersSquibb Company, Jacksonville, Florida). The nerve stimulator was tested for its function by applying the tip of the stimulator on the superficial facial nerve and monitoring the muscular activity. After this, the tip was applied directly over the

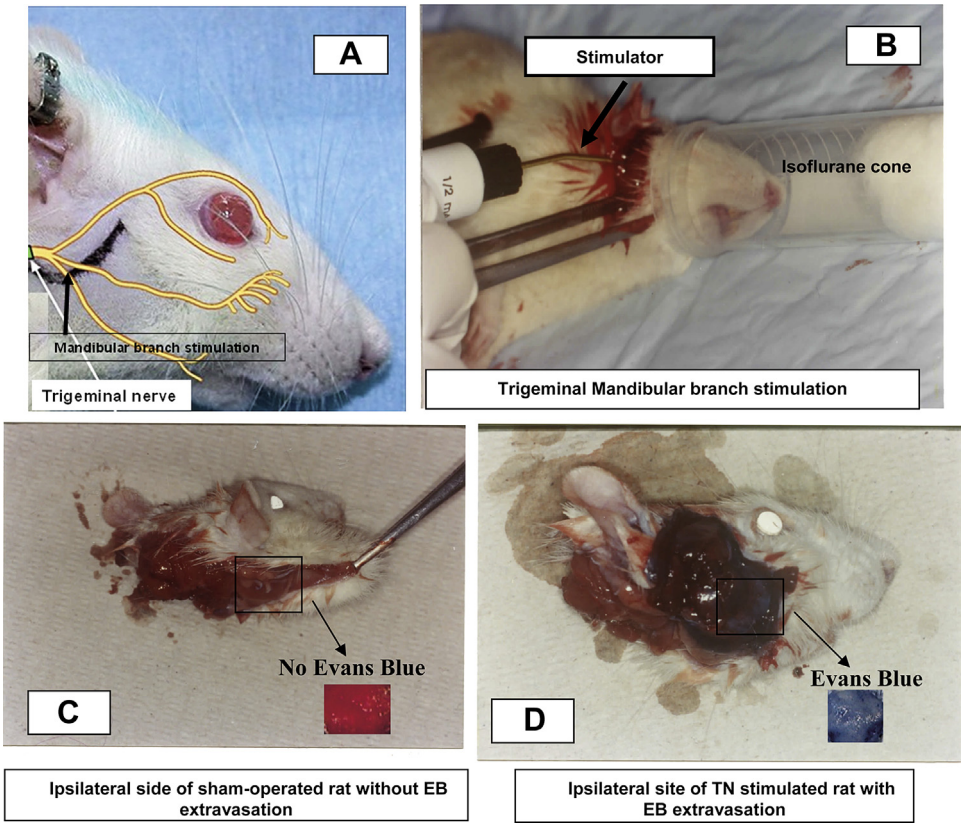

Figure 1. *(A)* Diagrammatic representation of how the mandibular branch of the trigeminal nerve was stimulated. *(B)* Photograph showing actual stimulation of an anesthetized rat. Photographs of the effect of trigeminal nerve stimulation on oral vascular permeability in *(C)* a sham-operated rat (red buccal mucosa) and *(D)* an experimental rat (dark-blue buccal mucosa indicating Evans Blue extravasation).

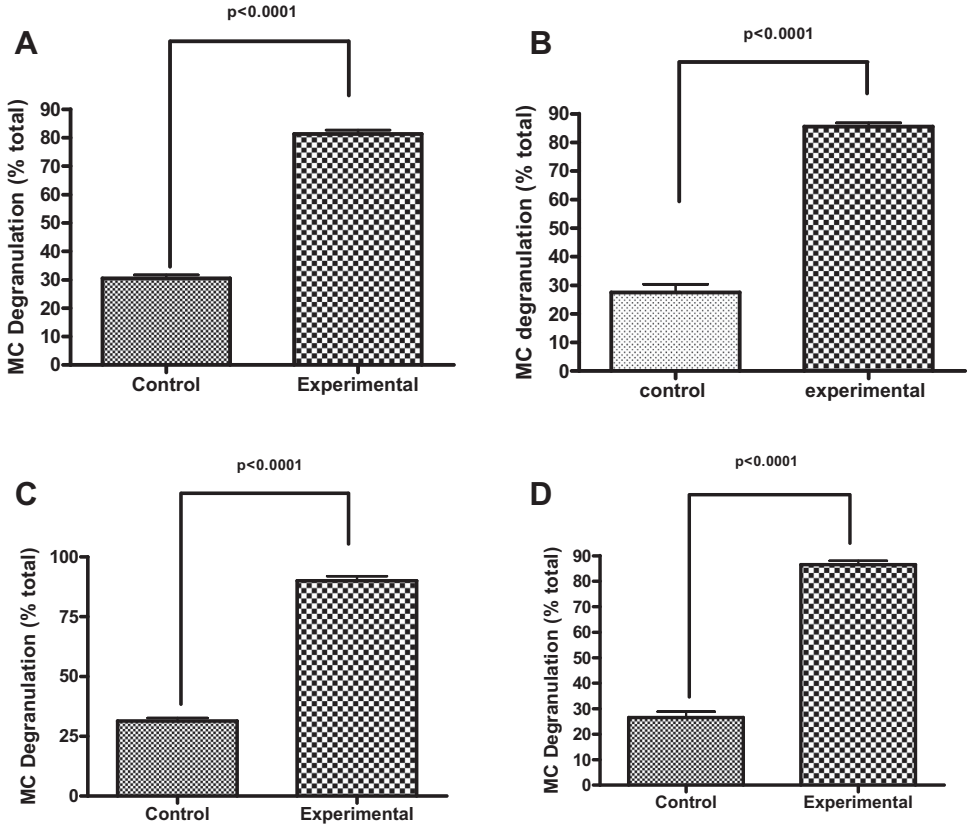

Figure 2. Effect of trigeminal nerve stimulation on mast cell (MC) degranulation in the *(A)* buccal mucosa, *(B)* tongue, *(C)* palate, and *(D)* gingiva.

exposed TN for 1 minute at 2.0 mV. The pin was placed subcutaneously to complete the electric circuit. In sham-operated rats (n = 3), the nerve stimulator was deactivated (no current was applied).

Cell Fixation and Staining for Light Microscopy

The tissue was cut into small pieces (0.5–1.5 μm thickness) and placed into fixative containing 3% glutaraldehyde, 2% paraformaldehyde, and 0.5% tannic acid in 0.1 mol/L of cacodylate HCl (pH 7.4). The tissue was fixed for 3 hours at room temperature. Thereafter, tissues were placed in fixative solution and left overnight at 4°C. Then, the tissues were washed with buffer solution (0.1 mol/L of cacodylate HC1, pH 7.4) 2 times and returned to the refrigerator for further processing.

Cell Fixation and Preparation for Transmission Electron Microscopy

Samples for ultrastructural analysis were processed as described previously.[24,25] Briefly, the tissue was fixed by immersion in 0.2 mL of modified Karnovsky fixative containing 6.8% sucrose (310 mmol/L; Fisher Scientific Co, Pittsburgh, Pennsylvania), 0.2 mol/L of Na-cacodylate buffer (pH 7.2; Laboratories, Plainview, New York), 2.0% paraformaldehyde (Fisher Scientific), and 2.5% glutaraldehyde (EM Sciences, Fort Washington, Pennsylvania). Samples were fixed in aldehyde suspension for 1 hour at room temperature, overlaid with 0.1 mol/L of Na-cacodylate buffer (pH 7.2), and left overnight at 4°C. The following day, samples were osmicated in 1% OSO_4 (Polysciences), washed, and overlaid with S-collidine buffer (pH 7.2; EM Sciences) in which they were left to stand overnight. Tissues were dehydrated through a graded series of ethanol (75%, 95%, and 100%) 2 times. The tissue was washed 25 minutes later at room temperature in absolute alcohol 1 time, left for another 25 minutes, and washed as before. Then, the tissue was treated with 100% propylene oxide for 10 minutes followed by a second 10-minute change of propylene oxide. The tissue was placed into open dishes containing 50 mL of Epoxy Embedding Solution EM Grade Epon 812 (Ladd Research Industries, Burlington, Vermont) with 50 mL of propylene oxide and then placed under the hood for overnight infiltration. Capsules containing embedded tissues were kept in a 56°C oven for 48 hours.

Sections 60 nm thick were cut with a diamond knife on an LKB 8801 microtome (LKB, Vienna, Austria) and mounted on uncoated 200 nm mesh copper grids. Sections were stained with uranyl acetate and lead citrate stains and examined with a Philips 300 transmission electron microscope (North American Philips Co, Mahwah, North Jersey).

Statistical Analysis

Twenty blocks of tissue were made from each site (tongue, buccal mucosa, palate, and gingiva) on the 2 sides (ipsilateral = experimental, contralateral = control) of each rat. There were 80 tissue blocks from each rat and 960 tissue blocks from 12 rats. From each tissue block, only 5 blocks were used randomly to produce sections 0.5 to 1.5 μm thick, which were stained with EB and fixed on glass slides. From 1 rat, 20 slides were made from the experimental side and 20 slides from the control side. The total number of slides examined from the 12 rats was 240. The total number of MCs and the percentage of MCs degranulated were calculated as: MC degranulation (%) = MCs degranulated (n)/MCs (N). Degranulation was present when (1) granule content was seen outside the periphery of the MC and (2) there was less than 50% MC staining with EB owing to the release of granule content.

For each tissue, the mean percentage of degranulated MCs was counted and compared between the ipsilateral and contralateral sides, between the ipsilateral sides of TN-stimulated and sham-operated rats, and between capsaicin- and vehicle-treated rats. Mean values were tested by the least significant difference test and by the nonparametric Mann-Whitney *U* test. Significance was set at a *P* value less than .05.

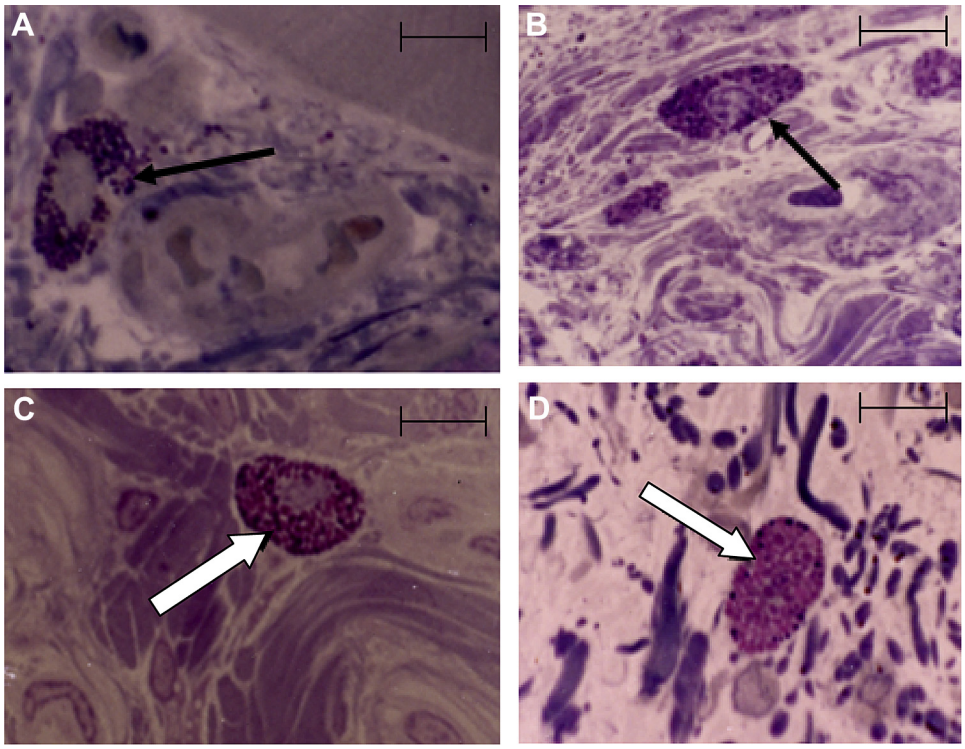

Figure 3. Light photomicrographs of mast cell degranulation in the buccal mucosa in the *(A)* control and *(B)* experimental sides and the tongue in the *(C)* control and *(D)* experimental sides. Intact (*black arrows*) and depleted (*white arrows*) secretory granules are displayed. Magnification bar = 10 μm.

Results

EB Extravasation

The TNS resulted in a marked increase of EB extravasation on the experimental side compared with the control side of the same rat. EB extravasation was evident as a dark color in the stimulated side at gross examination in each experiment (Fig 1C, D). Such a difference also was obvious between the experimental side of TN-stimulated and sham-operated rats (results not shown).

MC Degranulation

The total number of MCs was roughly equal in all tissue samples examined. In the experimental side, there was significantly ($P <$ .0001) increased degranulation of MCs in all 4 tissues examined (tongue, buccal mucosa, palate, and gingiva) compared with the contralateral side (Fig 1A–D) or the ipsilateral side of sham-operated rats (results not shown).

Mast cell degranulation was evaluated by histochemistry (Fig 2A, B) and confirmed by transmission electron microscopy (Fig 2C, D). The mean percentage of degranulated MCs in the buccal mucosa of the experimental side was 80 $\pm$ 4.7% compared with 31.5 $\pm$ 3.1% for the control side ($P <$ .0001; Fig 2A). The mean percentage of degranulated MCs in the tongue of the experimental side was 86.5 $\pm$ 3.7% compared with 26.4 $\pm$ 5.8% for the control side ($P <$ .0001; Fig 2B). The mean percentage of degranulated MCs in the gingiva of the experimental side was 81 $\pm$ 3.4% compared with 30.5 $\pm$ 2.9% for the control side ($P <$ .0001; Figure 2C) The mean percentage of degranulated MCs in the palate of the experimental side 85.5 $\pm$ 3.2% compared with 27.5 $\pm$ 7% for the control side ($P <$ .0001; Fig 2D).

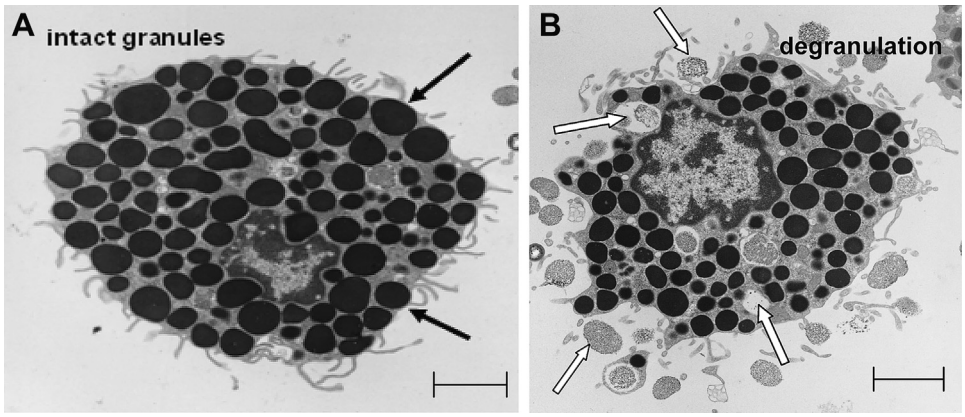

Figure 4. Transmission electron microscopic photomicrographs of the buccal mucosa showing mast cell degranulation in the *(A)* control and *(B)* experimental sides. Intact (*black arrows*) and depleted (*white arrows*) secretory granules are displayed. Magnification bar = 2 μm.

Capsaicin Treatment

To investigate the involvement of SP, some rats (n = 3) were treated neonatally with capsaicin to deplete SP, whereas others were treated with only vehicle (n = 3). TNS did not result in any statistically significant difference in EB extravasation in the experimental site compared with the vehicle-treated rats. There also was no statistically significant decrease in the mean percentage of degranulated MCs after TNS in the capsaicin-treated rats in the buccal mucosa (81.8 ± 7.4%), tongue (82.2 ± 5%), palate (79.5 ± 13.4%), or gingiva (88.04 ± 2.5%; Figs 3 and 4).

Discussion

The present findings showed significantly increased oral vascular permeability and MC degranulation after TNS. MC degranulation in the experimental side was noticeable in all 4 tissues. The nonspecific MC degranulation in the control sides might have been due to mechanical damage because MCs are sensitive to physical stimuli, such as the removal of buccal mucosa from surrounding tissues, which requires more mechanical manipulation than surgical removal of the tongue.[26] In addition, the cutting of tissues into small pieces results unavoidably in mechanical damage. Another possibility might be that some TN branches on the ipsilateral side might have crossed the midline and caused some MC degranulation contralaterally.

The purpose of injecting the rats with the EB was to investigate the effect of TNS on extravasation before proceeding to look at the status of MCs. Increased vascular permeability after TNS might be due in part to neuropeptides released from peripheral nerve endings acting on oral MCs.[27] Sensory neuropeptides, especially SP,[28] play an important role in neurogenic inflammation, including vasodilation, plasma extravasation, and recruitment of immune cells.[29] Capsaicin causes initial release and subsequent depletion of the content of SP from primary afferent neurons.[30] In fact, contrary to previous reports that SP is the major mediator involved in the degranulation of MCs after acute stress,[13] the present results showed that neonatal capsaicin pretreatment did not prevent MC degranulation. Neonatal capsaicin treatment might not have depleted all SP from the TN. Moreover, other neuropeptides, such as calcitonin gene-related peptide (CGRP), might be involved. TN fibers containing SP and immunoreactive CGRP have been described in the dental pulp, in the free and attached gingiva, the salivary glands, the tongue, and palatal mucosa in several species in addition to humans.[19] Human dental pulp cells, incubated in the presence of CGRP or SP, result in the release of interleukin-6, interleukin-1β, and TNF-α in a dose- and time-dependent manner.[31] Expression of CGRP has been reported to be significantly higher in inflamed human pulp tissue (eg, acute irreversible pulpitis) compared with healthy pulp tissue.[32,33]

The TN could be stimulated orthodromically by stress[3,30,34] or in retrograde fashion by oral triggers, such as bacteria, histamine, or inflammatory cytokines. For instance, acute stress has been shown to release CRH and neurotensin, which synergistically increase skin MC stimulation and vascular permeability.[22] Moreover, CRH could disrupt the intestinal epithelial barriers through MC release of proteases and TNF.[35] The oral mucosa may have a CRH-mediated regulatory system similar to the skin.[36] MC stimulation and increased vascular permeability could contribute to the pathogenesis of periodontal disease[37] and migraines.[38]

Owing to their unique properties, oral MCs[39,40] are ideally poised to serve as “gatekeepers” of the microvasculature in the oral cavity and participate with neuropeptides in the pathogenesis of oral inflammatory diseases.[37]

Trigeminal nerve stimulation resulted in significantly increased oral vascular permeability and MC degranulation only at the experimental side. Inhibition of MC degranulation may provide novel treatment approaches for oral inflammatory diseases.

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