Effect of inferior alveolar nerve axotomy on periodontal and pulpal blood flow subsequent to experimental tooth movement in rats

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The aims of this study were to evaluate the effect of inferior alveolar nerve (IAN) axotomy on periodontal (PDL) and pulpal blood flow incident to experimental tooth movement and to investigate whether nerve fiber regeneration coincides with blood flow changes. The first right mandibular molar was moved mesially for 3, 7, and 14 days after ipsilateral IAN axotomy in 29 rats. Four rats served as unoperated controls. At the end of each experimental period fluorescent microspheres (FM) were injected into the left ventricle and thereafter counted in serial sections in the PDL and pulp of the right and left first mandibular molars. The number of FM per tissue volume was taken as a measure of blood flow. Re-innervation of nerve fibers was mapped immunohistochemically 7, 14, and 21 days after IAN axotomy in 9 rats that had no orthodontic appliance. The statistical analysis showed no significant differences in the number of FM/mm^3 PDL between the denervated and the contralateral side at 3 and 7 days. At 14 days the PDL on the denervated side showed a significant increase in the number of FM/mm^3, coinciding with the initial periodontal nerve fiber re-innervation. In the pulp no significant differences were found between the denervated and the contralateral, innervated side in any experimental period. It can be concluded that IAN axotomy postpones an increase in periodontal blood flow until a sensory tissue re-innervation is established, thus indicating that neurogenic mechanisms play an important role in the development of the inflammatory reaction induced by experimental tooth movement. □ *Blood circulation; denervation; dental pulp; orthodontics; periodontal ligament*

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The periodontal ligament (PDL) and the pulp are well vascularized and densely innervated connective tissues. Histologic investigations have shown that periodontal and pulpal nerves are often observed in close association with, or in the vicinity of, blood vessels (1–3). These anatomic correlations have been proved to have functional implications, as both sensory and sympathetic fibers contain and release vasoactive neuropeptides that regulate local tissue blood flow (4, 5). In addition to this function, some of the sensory neuropeptides, such as calcitonin gene-related peptide (CGRP) and substance P (SP), are considered to be mediators of neurogenic inflammation in various human and animal tissues (6–8).

Neurogenic inflammation is a reaction characterized by changes in the microcirculation, elicited by mechanical (9), chemical (10), and/or electric (11, 12) stimulation of sensory nerve fibers. Activated afferent nerve endings release neuropeptides directly and via axon reflex mechanisms and trigger an inflammatory response that is, however, abolished after sensory denervation or depletion of neuropeptides (9, 13).

It has been hypothesized that the application of any type of orthodontic force leads to activation of PDL mechanoreceptors and release of stored neurotransmitters that interact with dental tissues peripherally (14). Extensive experimental evidence confirms that dental tissue reactions after orthodontic tooth movement are inflammatory in nature and comprise increased periodontal and pulpal blood flow (15, 16), periodontal vasodilatation with plasma extravasation (17), erythrocyte diapedesis (18), and an increased number of periodontal tissue channels (19). It has also been shown that, at inflammation sites, CGRP- and SP-containing nerve fibers show a concomitant increase in fiber density and intensified immunoreactivity (20, 21), thus supporting the hypothesis that neurogenic mechanisms may play an important part in the development of dental tissue inflammation incident to experimental tooth movement.

In view of these observations it can be assumed that deprivation of sensory innervation in the PDL and pulp will lead to a changed inflammatory response once orthodontic forces are applied. Therefore, the main purpose of this study was to evaluate and semiquantify the effect of inferior alveolar nerve (IAN) axotomy on blood flow changes in the PDL and pulp after experimental tooth movement. Furthermore, it was of interest to investigate whether changes in tissue blood flow coincided with the re-innervation of periodontal and pulpal nerve fibers containing CGRP and neuropeptide Y (NPY).

Materials and methods

Animals

A total of 42 Mol:WIST male rats, weighing approxi-

mately 200 g, were used in this study. The experimental group comprised 29 operated animals, whereas 4 animals served as an unoperated control group. Nine rats were prepared for immunohistochemical investigation of the nerve fiber re-innervation. All animals were housed in polycarbonate cages in a conventional animal room and fed standard pellet diet with water ad libitum. All experiments were registered and approved by the Norwegian Experimental Animal Board (NEAB).

Experimental procedure

The operations were carried out under general anesthesia, for which purpose a subcutaneous injection of fentanyl/fluanisone (Hypnorm®) and midazolam (Dormicum®), 0.2 ml/100 g body weight, was used. As previously described (16), an orthodontic appliance consisting of a coil spring ligated to the first right mandibular molar and connected to the mandibular incisors was inserted in the animals of the experimental group. The coil exerted a force of approximately 0.5 N. During the experimental periods the force attenuated, and by day 14 the coil was inactive. Immediately after the insertion of the appliance, unilateral axotomy of the right IAN was done as described earlier (22). A horizontal skin incision was made on the lateral aspect of the mandible. The masseter muscle was bluntly dissected, thus gaining access to the bone surface right above the mandibular foramen. The overlying bone was removed with a dental bur, and the exposed alveolar nerve was elevated and then sectioned with scissors, leaving the blood vessels intact. The incision was closed with three sutures (Supramid, 5/0), and the animals were allowed to recover. The left, contralateral side served as an intragroup control. After the experimental procedures the animals received daily doses of analgesic for 2–3 days (buprenorphine (Temgesic®), 0.1 ml/100 g, twice a day) and were fed a soft diet. All animals were weighed before the start of the operation and before being killed. Despite the overall uneventful recovery, the rats in the 3-day group ($n = 9$) lost approximately 8% of their weight. However, the rats in the 7-day ($n = 10$) and 14-day ($n = 10$) groups gained weight, the latter showing an average increase of 30%.

Three, 7, and 14 days after the operation, the animals were re-anesthetized, and 0.2 ml fluorescent microspheres (FM) (Fluoresbrite Plain Microspheres; diameter, 9.6 μm; Polysciences, Inc., Warrington, UK) suspended in 10% Ficoll-70 in 90% saline were injected into the left ventricle (16). Two minutes after the injection the rats were decapitated, the jaws excised, fixed in 4% paraformaldehyde for 24 h, and then demineralized in 10% ethylenediaminetetraacetic acid (EDTA) plus 7.5% polyvinylpyrrolidone for 4 weeks.

The animals in the control group received no orthodontic appliance. They were injected with FM in the same manner and underwent the same procedures as described for the experimental group.

An additional group of nine animals was prepared for immunohistochemical mapping of the nerve fiber reinnervation. They had only axotomy of the IAN and received no orthodontic appliance. Seven ($n = 3$), 14 ($n = 3$), and 21 ($n = 3$) days after the axotomy they were re-anesthetized, and a transcardiac perfusion was performed with phosphate buffer containing 0.03% heparin, followed by 4% paraformaldehyde and 0.2% picric acid in 0.1M phosphate buffer, pH 7.4. The demineralization procedure was the same as for the animals injected with FM.

After demineralization the jaws were sectioned sagittally at 40 μm in a cryostat microtome. Serial sections for evaluation of blood flow were examined unstained in a fluorescent microscope. Sections used for evaluation of the nerve fiber regeneration were alternately incubated for 72 h in antibodies against CGRP (1:7500) and NPY (1:4000, Cambridge Research Biochemicals, Cambridge, UK). Antigen–antibody complexes were detected with the avidin-biotin-peroxidase (ABC) method, using a comercially available kit (Vectastain ABC kit, Vector Laboratories, Burlingame, Calif., USA) and visualized by 3'3 diaminobenzidine (DAB) in the presence of 0.2% $(NH_4)_2Ni(SO_4)_26H_2O$ to enhance the immunostaining. Finally, the sections were stained with methylene blue/azure II,

Table 1. The mean number and standard deviation (*s*) of fluorescent microspheres (FM) per mm^3 periodontal ligament and pulp in the control group, in the left, contralateral, and the right, denervated, first mandibular molar at 3, 7, and 14 days after inferior alveolar nerve axotomy and insertion of an orthodontic appliance

			Periodontal ligament		Pulp	
Experimental period (days)	No. of animals		Mean	*s*	Mean	*s*
	4	Control group	219.3	55.3	145.4	25.6
3	9	Contralateral molar	228.5	82.5	137.3	47.7
		Denervated molar	265.7	87.8	144.0	63.5
7	10	Contralateral molar	190.6	74.3	91.0	38.7
		Denervated molar	237.4	86.4	96.3	50.1
14	10	Contralateral molar	220.8	111.4	138.5	47.0
		Denervated molar	339.7*§	107.3	140.9	30.7

* Significance, $P \leq 0.01$ of Wilcoxon sign-rank test between the left, innervated, and right, denervated, molar.
§ Significance, $P \leq 0.05$ of Mann–Whitney test between the control and the denervated molar.

coverslipped, and analyzed in a light microscope. The specificity of the immune reaction was tested by omitting the primary or secondary antibody and replacing it with phosphate-buffered saline (PBS). No specific immunolabeling was observed in these sections.

Evaluation procedure

The diameter of the FM was 9.6 ± 0.8 μm, which means that most would be trapped in the precapillary arterioles (23). FM were counted on every section throughout the periodontal and pulpal tissues of the right and left first mandibular molars, summarized, and then divided by the number of sections. The mean number of FM per section was then multiplied by the mean number of sections per molar, to obtain the total number of FM per tissue volume. The number of FM/mm^3 PDL and pulp was taken as a relative measure of blood flow (16). The mean tissue volume of the PDL (2.49 mm^3) and pulp (2.16 mm^3) of the mandibular first molar was calculated by the use of Cue-3 Image Analizer (Galai Production, Migdal Haewek, Israel).

Since the denervation procedure was always done on the right side and the right first mandibular molar was always the tooth undergoing experimental tooth movement, in the further text, the right molar is referred to as the operated or denervated molar, and the left one as the unoperated or contralateral first mandibular molar.

The Wilcoxon sign-rank test was used to assess the differences in the number of FM/mm^3 PDL and pulp tissue between the right and left first mandibular molars within the experimental and control groups. The Mann–Whitney test was used for intergroup comparison or to evaluate the differences in the number of FM/mm^3 tissue between the control group and the denervated molar and between the control group and the contralateral first mandibular molar.

All immunohistochemically labeled sections of the unoperated contralateral and denervated first mandibular molar were carefully examined under light microscopy. The density of the CGRP- and NPY-containing nerve fibers in the PDL and pulp of the denervated side was evaluated as 'less than' and 'equal to' the contralateral, innervated sections (21). Their morphology and distribution were evaluated qualitatively.

Results

Control group

The statistical analysis showed no significant differences in the mean number of FM/mm^3 PDL and pulp between the left and right first mandibular molars. Therefore, the number of FM from each side was pooled, and their mean values (219.3 ± 55.3 for the PDL and 145.4 ± 47.7 for the pulp) were used when comparing the control group with the experimental group (Table 1).

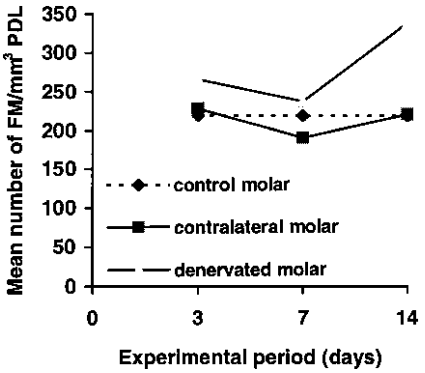

Fig. 1. The mean number of fluorescent microspheres (FM)/mm^3 periodontal ligament (PDL) of the controls and during experimental tooth movement in the left, contralateral, and the right, denervated, first mandibular molar 3, 7, and 14 days after inferior alveolar nerve axotomy.

Experimental group

Periodontal ligament. The mean number of FM/mm^3 PDL of the unoperated mandibular molar showed rather even distribution throughout the various experimental periods without significant differences when compared with the control molar (Table 1, Fig. 1). The PDL of the denervated first mandibular molar showed similar distribution of FM/mm^3 tissue at 3 and 7 days, giving values closely following the contralateral side (Table 1). However, after a slight, insignificant decrease at 7 days, the mean number of FM/mm^3 PDL on the experimental side showed a significant increase at 14 days when compared with the PDL of both the contralateral and the control first mandibular molar (Table 1, Fig. 1).

Pulp. The distribution and mean number of FM/mm^3 pulp were very similar for the unoperated and the denervated first mandibular molars and also for the controls. After 7 days a slight decrease in the number of

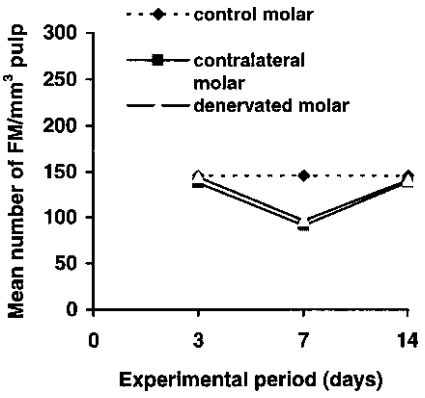

Fig. 2. The mean number of fluorescent microspheres (FM)/mm^3 pulp of the controls and during experimental tooth movement in the left, contralateral, and the right, denervated, first mandibular molar 3, 7, and 14 days after inferior alveolar nerve axotomy.

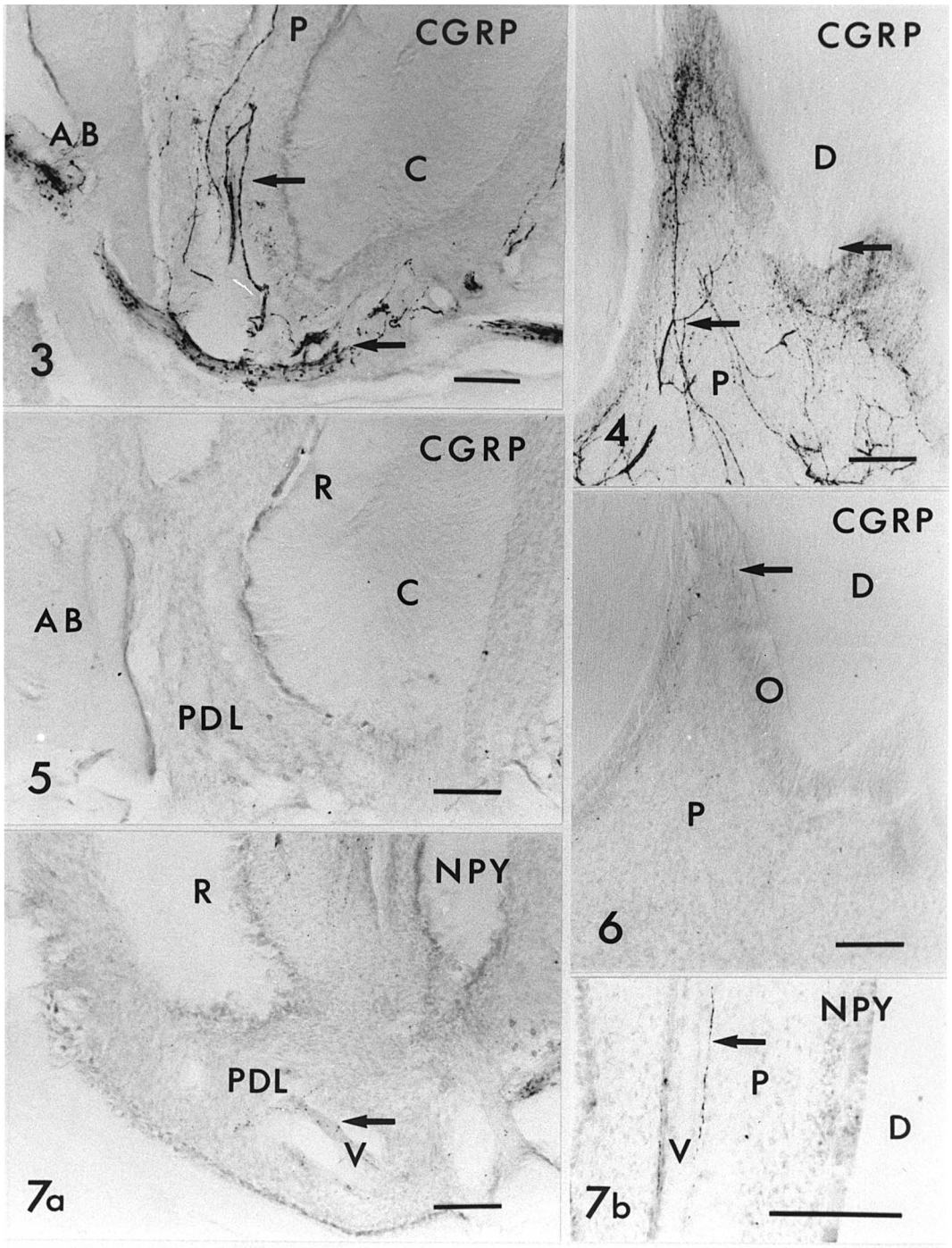

Fig. 3. Periapical area of a distal root of an unoperated, contralateral first mandibular rat molar. The distribution of calcitonin gene-related peptide (CGRP)-immunoreactive (IR) nerve fibers (arrows) in the periodontal ligament (PDL) and the apical pulp (P) is shown. AB = alveolar bone; C = cementum.
Fig. 4. Distal pulp horn of an unoperated contralateral first mandibular molar densely supplied by CGRP-IR nerve fibers (arrows) in the pulp (P) and dentin (D).
Fig. 5. Periapical area of a distal, first mandibular molar root (R). Seven days after inferior alveolar nerve (IAN) axotomy the PDL is still almost devoid of CGRP-immunopositive nerve fibers.
Fig. 6. Distal pulp (P) horn of a first mandibular molar. Seven days after IAN axotomy a few, tiny CGRP-IR nerve fibers (arrows) are seen in the odontoblast area (O).
Fig. 7. Periapical area and root pulp (P) of the first mandibular molar, distal root (R). Seven days after IAN axotomy several NPY-IR nerve fibers (arrows) are found around large blood vessels (V) in the PDL (a) and in the root pulp (b).
Scale bars = 0.1 mm.

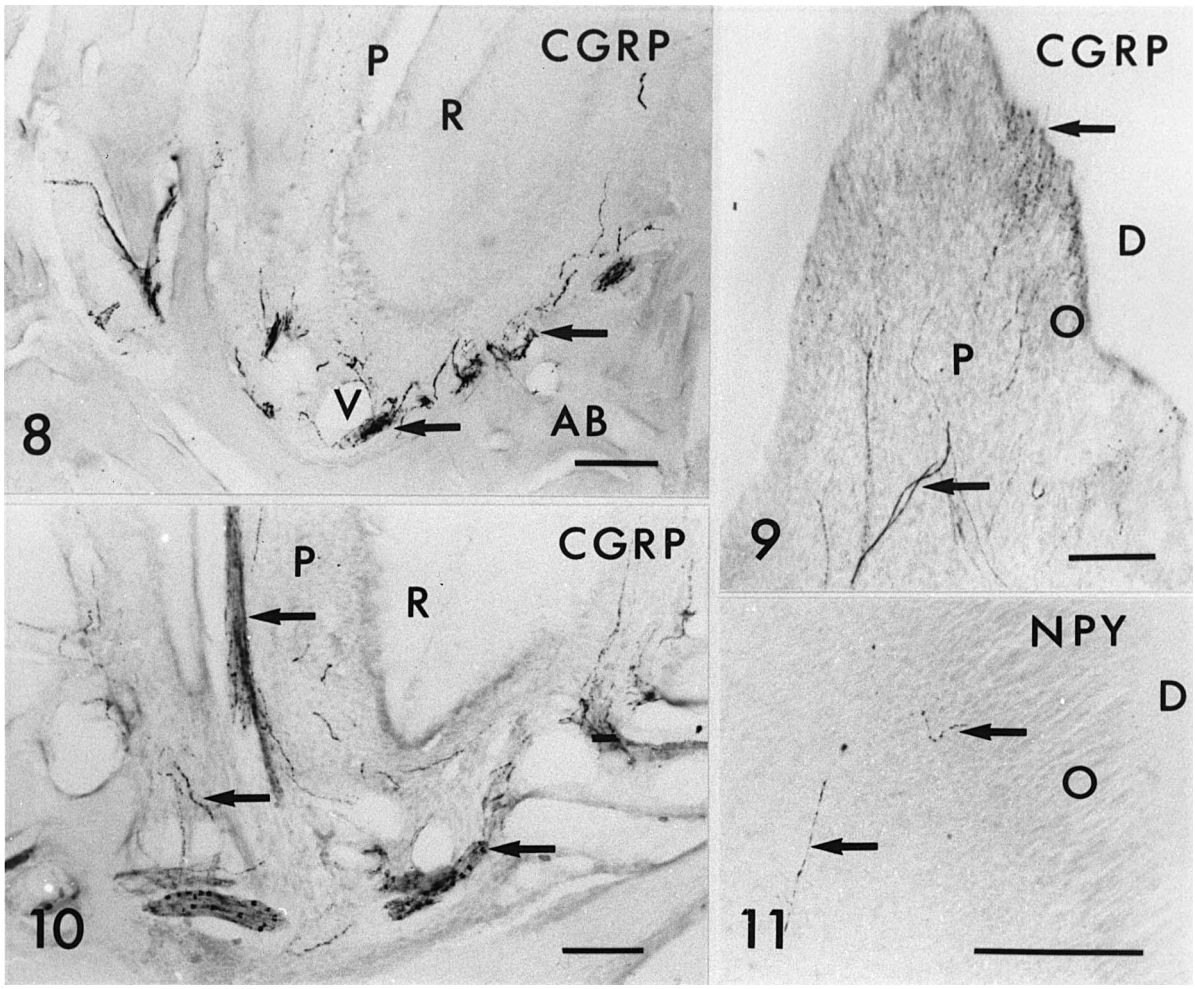

Fig. 8. Periapical area of the distal root (R) of the first mandibular rat molar. Fourteen days after inferior alveolar nerve (IAN) axotomy, re-innervating calcitonin gene-related peptide (CGRP)-containing nerve fibers (arrows) are frequently seen close to blood vessels (V) in the periodontal ligament (PDL). AB = alveolar bone; P = pulp.
Fig. 9. Distal pulp (P) horn of a first mandibular molar. Fourteen days after IAN axotomy several re-innervating CGRP-immunopositive nerve fibers (arrows) are seen in the main pulp (P), odontoblastic area (O), and, occasionally, in the dentin (D).
Fig. 10. Periapical area of the distal root (R) of the first mandibular molar, 21 days after IAN axotomy. The density of re-innervating CGRP-immunoreactive (IR) nerve fibers (arrows) in the PDL has increased but is still reduced compared with the contralateral side (Fig. 2). The root pulp (P) is extensively supplied by CGRP-IR fibers.
Fig. 11. Distal pulp horn 21 days after IAN axotomy. NPY-IR nerve fibers (arrows) are seen close to and within odontoblastic layer (O). Scale bars = 0.1 mm.

FM/mm^3 was found on both sides, to be followed by an insignificant increase at 14 days (Fig. 2). No significant differences in the number of FM/mm^3 tissue was found either between the contralateral and the denervated pulp or between the control and denervated pulp (Table 1).

Immunoreactive nerves

The distribution of CGRP- and NPY-containing nerve fibers in the PDL and pulp on the innervated side (Figs. 3 and 4) was in accordance with previous investigations (21, 24).

7 days. Both the PDL and the pulp on the denervated side were almost completely devoid of nerve fibers containing CGRP (Figs. 5 and 6). Occasionally, tiny, individual nerve fibers could be seen in the apical PDL and in the coronal pulp. NPY-immunoreactive nerves were mainly found around blood vessels in the apical PDL (Fig. 7a) and in the root pulp (Fig. 7b), although at a reduced density compared with the controls.

14 days. On the axotomized side the density of periodontal and pulpal CGRP-labeled nerve fibers was still less than on the contralateral side, although the re-innervation of the PDL showed more progress than the pulp. The periapical region of the PDL on the denervated side (Fig. 8) showed immunoreactive fibers that still lacked

a regular arrangement, even though they were mainly found close to blood vessels. CGRP-immunoreactive nerve fibers could be seen in the distal root pulp but were less frequent in the mesial one. Correspondingly, the distal pulp horn showed many immunopositive CGRP fibers that even reached the odontoblastic layer (Fig. 9), whereas few nerve fibers were found in the medial and mesial pulp horns. In contrast, NPY-immunoreactive nerves were often seen both in the mesial and distal parts of the root and coronal pulp and in the apical area of the PDL, mainly situated proximal to blood vessels.

21 days. Periodontal CGRP-immunopositive nerve fibers began approaching the density of the controls, predominantly in the periapical area (Fig. 10). There were still fewer pulpal nerves immunoreactive to CGRP than on the control side, although the density in the medial and mesial pulp horns was increased as well. NPY-immunopositive fibers reached the level of the control periodontal and pulpal nerves, occasionally being present close to the odontoblastic layer (Fig. 11).

Discussion

The results of this study show that mandibular rat molars show a delayed but significant increase in periodontal blood flow 2 weeks after ipsilateral IAN axotomy and application of orthodontic force, compared with the contralateral side and with previous studies. This increase coincides with the initial re-innervation of the PDL, indicating that neurogenic mechanisms play an important part in the control of periodontal blood flow during experimental tooth movement.

It is well established that short- and long-term experimental tooth movement induces vascular, inflammatory reactions in the PDL and pulp (16, 17, 25). We have previously shown that innervated maxillary rat molars undergo a significant increase in periodontal and pulpal blood flow as early as 3 days after the application of orthodontic force, reaching its peak at 7 days (16). At later stages, 14 and 21 days, periodontal and pulpal blood flow decreases and finally approaches control levels. The enhanced blood flow changes coincide with an increase in CGRP-immunoreactive nerve fiber density in the corresponding tissues (20, 21), thus suggesting a functional relationship between the nerves and the vasculature. It can therefore be presumed that tissue denervation is followed by a subsequent decrease in blood flow, but as the process of re-innervation develops, a gradual blood flow increase would be expected. In the initial experimental stages after denervation, we found no significant changes in periodontal blood flow between the denervated and the contralateral side. However, 14 days after the beginning of experimental tooth movement the PDL on the denervated side showed a significant increase in blood flow, concurring with the initial re-innervation of CGRP-containing nerve fibers in the PDL. Being in concordance with the results of re-innervation studies in rat oral tissues (26, 27), these results confirm that nerve fiber regeneration is clearly evident 14 days after IAN axotomy, and, thus, a concomitant increase in blood flow and tissue inflammatory response is fully expectable.

Considerable evidence exists that peptidergic sensory neurons initiate and mediate neurogenic inflammation in different tissues (6, 7, 8) including the PDL (9) and pulp (28). This process, comprising vasodilatation followed by increased tissue blood flow and plasma extravasation, can be completely inhibited by blocking the sensory nerve terminals or by surgical and chemical denervation (9, 13). In our experiment cutting of the IAN deprived the PDL and pulp of their main sensory supply and hence of the ability to develop a neurogenic inflammatory reaction on activation by mechanical stimuli. The significant increase in periodontal blood flow after re-innervation of the sensory, CGRP-containing fibers lends evidence to the assumption that neurogenic components are closely involved in the development of inflammatory tissue responses subsequent to experimental tooth movement.

The re-innervation of the PDL showed greater progress than the re-innervation of the pulp and thus supports previous findings (26). This is probably owing to the difference in their anatomic configuration, which enables the PDL to receive, in addition to regenerated IAN fibers, many collateral sprouts from the ipsilateral lingual nerve through the lateral alveolar foramina (29). The pulp, in contrast, receives its nerve supply mainly through the apical foramen either from the IAN or from the collateral nerve fibers or from both sources. It has been shown that pulpal hemoregulatory functions do not return to normal levels even after nerve regeneration (30) and that regenerated IAN fibers supplying the pulp have slower than normal conduction velocities (29). The later and sparse re-innervation of the target tissue and a reported incomplete function in newly formed nerve sprouts can explain why we found no increase in pulpal blood flow 2 weeks after IAN axotomy, as opposed to the PDL. Another explanation might be that the applied orthodontic force had diminished to a point where it did not have enough impact on the pulpal tissue to induce significant vascular, inflammatory response. We have previously shown that experimental orthodontic force has a longer-lasting influence on blood flow in the PDL than in the pulp (16).

The present findings show that both periodontal and pulpal blood flow values were higher 3 than 7 days after axotomy. This drop in blood flow, although insignificant, may indicate that oral tissues, in case of compromised sensory nerve supply, possess other local mechanisms that meet their immediate demands. Endothelial cells react to mechanical force as well as to various neurohumoral mediators by producing several vasoactive substances that regulate the vascular tone of the underlying smooth-muscle cells (31). Nitric oxide, for example, is shown to be a powerful, short-lived vasodilator, whereas endothelins exert a prolonged vasoconstrictive effect (31). Activation and production of endothelin-derived relaxing and constricting factors can be induced by, among other

substances, interleukins (32). This suggests that mononuclear phagocytes, being the major cell source for interleukin production, are indirectly involved in the control of local tissue blood flow. We have shown that experimental tooth movement leads to increased recruitment of mononuclear phagocytes in the PDL (33) and thus confirmed their involvement in the process of inflammatory tissue reactions.

Sympathetic nerve fibers are shown to control cat PDL blood flow for vasoconstriction (4), but their role in neurogenic inflammation is unclear. In this study we found that 7 days after IAN axotomy, sympathetic, NPY-containing nerve fibers were reduced in density but not completely eliminated. Later on their density in the PDL and pulp increased, and NPY-positive fibers were seen close to the odontoblastic layer. It has been acknowledged that large to medium-sized sensory neurons of the trigeminal ganglion upregulate and express NPY after IAN injury and that NPY colocalizes with CGRP in regenerated pulpal fibers (34). Furthermore, NPY has been shown to exert inhibitory influence on sensory nerve-derived vasodilatation in the rat pulp (35) and rat mesenteric vascular bed (36). Therefore, the possibility cannot be excluded that the increased content of NPY in the pulp might be the reason for a missing blood flow increase even after sensory tissue re-innervation.

It can be concluded that IAN axotomy suspends the increase of periodontal blood flow, and hence the development of an appropriate inflammatory reaction, until a sensory tissue re-innervation is achieved. This indicates that neurogenic mechanisms play an important part in the development and control of the inflammatory tissue responses subsequent to experimental, orthodontic tooth movement.

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