Investigation of the role of endotoxin in periapical inflammation

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Dental pulps in two dogs were removed and the canals shaped in six matched pairs of roots. Canals on the experimental side were injected with *Salmonella minnesota* R-595 endotoxin and canals on the contralateral control side were injected with saline solution at weekly intervals. Radiographic examinations were performed weekly, and histologic evaluations were made at four to five weeks. Periapical radiographic changes occurred sooner and to a more severe degree with the roots containing endotoxin than with the roots containing saline solution. Histologic evaluation showed greater periapical bone destruction and a more marked inflammatory response. Polymorphonuclear leukocytes were the predominating cells. The results of this study indicate that endotoxins may have a role in periapical inflammation and bone destruction.

Gram-negative bacteria are frequently isolated from root canals of necrotic teeth.1-4 The outermost membrane of the gram-negative cell wall contains endotoxin, a lipopolysaccharide complex, which is released on the death of the cell. Endotoxins have potent and diverse biological properties, and they are potent inflammatory agents. Simon and others⁵ found a statistically significant correlation between the quantity of endotoxin in gingival exudate and the clinical degree of gingival inflammation. Morrison and Kline⁶ showed activation of the classic and alternative pathways of complement by bacterial endotoxins, which is not dependent on antibody to the endotoxins. Complement activation releases biologically active peptides, which mediate a number of aspects of the inflammatory process, such as enhancement of vascular permeability

and chemotactic attraction of polymorphonuclear leukocytes (PMNs) and macrophages.⁷ Lysosomal enzymes released by PMNs during phagocytosis⁸ are capable of soft and hard tissue destruction. Complement activation has also been shown to cause bone resorption in vitro because it enhances prostaglandin synthesis.⁹

Exposure of human leukocytes to

generate a substance that is capable of forming kinins,¹⁰ which cause increased capillary permeability, hypotension, and pain.¹¹ Interaction of endotoxin with serum results in the degranulation of mast cells, which are abundant sources of histamine and heparin.⁷ Significant numbers of mast cells have been found in periapical

endotoxin in vitro has been shown to



Fig 1—Preoperative radiograph of mandibular right third and fourth premolars.



Fig 2—Photomicrograph of unperforated root apex. RC, instrumented root canal; ap, microscopic apical ramifications of root canal; pdl, periodontal ligament with scattered PMNs; d, dentin; c, cementum (H&E, orig mag ×31).

granulomas.^{12,13} Histamine is a potent inflammatory agent that enhances vascular permeability and has been shown to synergistically inhibit bone growth in vitro when combined with endotoxin.¹⁴ Morrison and Cochrane¹⁵ demonstrated that purified endotoxin can directly activate the Hageman factor (Factor XII), initiating the intrinsic clotting system.

Endotoxin has a mitogenic effect on B lymphocytes of mice and guinea pigs and has stimulated guinea pig B lymphocytes to produce a factor chemotactic for macrophages. Endotoxin can activate macrophages, resulting in the production and release of significant quantities of collagenase.¹⁶ Collagenase has been found in several chronic inflammatory lesions including periodontal disease.^{17,18}

Endotoxins are extremely potent antigens and are T cell-independent in that they can stimulate antibody formation without the help of T lymphocytes.¹⁹ They elicit predominantly an

IgM response, a pentavalent type of antibody, which would be expected to be more effective in tightly binding, capturing, and focusing the antigen for initiating the immune response than the smaller IgG antibody, mainly elicited by protein antigens.²⁰ Dahlen and Fabricius²¹ experimentally infected root canals of monkeys with a gramnegative oral organism, Bacteroides oralis, and showed that serum antibodies to the endotoxin were extracted from that same organism six months later. Antibody binding to endotoxin could result in antigen-antibody binding complexes capable of activating complement,⁷ and thus generate mediators of inflammation and tissue destruction.

Lymphocyte transformation, one of the in vitro indicators of cell-mediated immunity,22 has been induced in human lymphocytes with endotoxins.^{23,24} Stimulated lymphocytes secrete soluble mediators of inflammation (lymphokines), which have many actions including chemotaxis of PMNs and macrophages, migration inhibition and activation of macrophages. and cytotoxicity.11 Horton and others25 have demonstrated that a product of stimulated human peripheral blood leukocytes, called osteoclast-activating factor, induces osteoclastic resorption of fetal bone in organ cultures.

Purified endotoxins have stimulated osteoclastic bone resorption in tissue cultures of fetal rat bones.²⁶ This resorption was potentiated by the addition of heparin. Hausmann and others²⁷ found the lipid A portion of the endotoxin molecule to be primarily responsible for the in vitro bone resorption by a number of endotoxin preparations. Endotoxin extracted from an oral organism, *B melaninogenicus*, in concentrations as low as 0.1 mg/ml has stimulated resorption of fetal rat bone in tissue culture.²⁸ Robinson and Shapiro²⁹ have shown a significant depression of bone cell respiration that was directly proportional to the concentration of endotoxin.

Schein and Schilder,³⁰ in a study assaying fluid aspirated from root canals in humans by the limulus lysate technique, found that pulpless teeth contained significantly greater concentrations of endotoxin than those with vital pulps, and those that had periapical radiolucencies had significantly higher levels than those with none. They suggested that endotoxins stimulate bone resorption in vivo as well as in vitro. A positive correlation of increased endotoxin concentration in the root canal with clinical symptoms was also found. It was postulated that the release of endotoxins from gramnegative organisms in the canal or at the periapex could be a significant factor in acute periapical inflammation. The results of Sundqvist and others³¹ suggest that purulent periapical inflammation in certain cases may be induced by specific combinations of bacterial species in the root canal and that the presence of B melaninogenicus or B asaccharolyticus, both containing endotoxin, is essential in such combinations.

The purpose of this study is to evaluate the effect of endotoxin on periapical tissue by introducing endotoxin into the root canals of dogs.

METHODS AND MATERIALS

The endotoxin preparation used in this study was derived from the heptose-deficient rough mutant *S* minnesota R-595. It was extracted by the phenol-chloroform-petroleum ether procedure described by Galanos and others³² and was supplied in lyophilized form. The endotoxin was subse-



The saline solution, used as a control and diluent for the lyophylized endotoxin, was prepared from distilled water and sodium chloride to a concentration of 0.85% and then autoclaved. Glassware for the solutions was sterilized with dry heat at 160 C for three hours. Samples of all endotoxin and saline solutions were streaked on blood agar plates and incubated for 48 hours at 37 C as a control for contamination by microorganisms.

Two adult mongrel dogs, weighing 22.7 and 25.0 kg, were premedicated with an intramuscular injection of 1.5 ml Ketaset. They were then anesthetized intravenously with sodium pentobarbital, 60 mg/ml (Nembutal sodium).

The study used mandibular third and fourth premolars bilaterally. The teeth were examined radiographically to ensure that the root apexes were mature and to rule out pre-existing abnormalities of hard tissues. Clinical examination was performed to eliminate the possibility of sinus tract stomas, swellings, and significant periodontal disease.

Canine third and fourth premolars each have two roots, one mesial and one distal (Fig 1). Each root has a single canal. The main canal is not continuous to the apex, but ends approximately 2 to 3 mm from the radiographic apex by branching into multiple, almost microscopic ramifications (Fig 2). These premolars are the only teeth whose surrounding structures permit the radiographic film to be placed parallel to the long axis of the tooth so that a paralleling technique could be used, and which are not too far posterior to prevent reasonable



Fig 3—A, photomicrograph of perforated apex with saline solution. MC, mandibular canal; f, compressed fibrous connective tissue; pdl, widened periodontal ligament with fibrosis across foramen; inf, mild inflammatory tissue extending into root canal. B, perforated apex with endotoxin; res, root resorption; les, periapical lesion with moderate inflammation extending into root canal; inf, narrow extension of inflammation and edema into mandibular canal (MC) (H&E, orig mag $\times 31$).

access for the root canal procedures. Each canal could be treated as a separate experimental site, thus allowing up to eight sites or four matched pairs per dog.

Reproducibility of radiographic angulation and source-to-film distance was accomplished by using a posterior film-holding instrument with an aiming device (Rinn Corp). The instrument was held in the exact position each time by closing the teeth into custom-made occlusal indexes fabricated from cold-curing acrylic resin. The assembly or jig was firmly held in position by attaching a stiff rubber band around the dog's snout. Use of a standard size dental periapical film permitted all four roots of the third and fourth premolars to be included on one film (Fig 1). All radiographs in this study were taken with a tube head (Phillips Oralix) with an output of 50 kVp and 7.5 mA. Standardized time, temperature, and solutions were used to process the films.

Preoperative radiographs were taken before isolation of the teeth with a rubber dam and disinfection of the field with 70% isopropyl alcohol. Aseptic techniques were used throughout the procedure. The pulp chamber and canal orifices were exposed with sterile fissure and round burs. Length of tooth was determined by placing a K-file with silicone stop in the canal to the level of apical arborization and by confirming the level with a radiograph. The pulp tissue was then removed with barbed broaches. All canals (six matched pairs) were reamed to the level of the apical arborization with K-files to a size two sizes larger than the first instruments to cut clean dentin at the apex. The coronal aspect was then circumferentially filed with Hedstroem files. Irrigation during canal preparation was performed with copious amounts of sterile, nonpyogenic saline solution.

Four of the matched pairs (two each in dogs 1 and 2) were then perforated with sterile engine-driver Gates-Glidden drills, sizes 1 and 3, through the remaining tooth structure to the apex. An apical perforation length was first



determined from the known instrument length to the apical arborization. Complete perforation was confirmed radiographically with a K-file in the canal extending just beyond the radiographic apex. All canals were subsequently dried with measured sterile paper points to prevent further periapical trauma, after which 0.1 ml of the appropriate solution was injected into each canal with a sterile 23-gauge, 1-inch disposable hypodermic needle attached to a 1-ml disposable syringe.

The four matched pairs of roots in which apexes were perforated were injected with the endotoxin suspension on the experimental side and saline solution on the control side. The endotoxin suspension was vigorously shaken before it was drawn into the syringe. An effort was made to insert the needle as far into the canal as possible without penetrating the apical opening.

Two matched pairs (both in dog 2)

were instrumented to the level of the apical arborization, but not perforated to the apex. One of the unperforated pairs was injected with endotoxin on the experimental side and saline solution on the control side, whereas, the other was just instrumented and dried as a control.

Small plugs of sterile cotton were then placed into the coronal aspect of all canals over which zinc oxide-eugenol cement was applied. After the cement had set, amalgam was condensed into the chamber and carved flush with the flattened crown. Postoperative radiographs were taken immediately.

At approximately one-week intervals (Table), the teeth were radiographed, and the canals were reopened to inject 0.1 ml of fresh solutions. The canals were again sealed as before. The control pair that had been instrumented only was not disturbed.

At the end of the experimental peri-

ods (four weeks for the perforated teeth and five weeks for the unperforated), the teeth were radiographed and the dogs were killed with an intravenous injection of an euthanasia agent (T-61). Block sections of the mandibles containing the test teeth were removed and fixed in 10% neutral buffered formalin. The specimens were then decalcified in Kristensen's fluid,33 embedded in paraffin, sectioned in a buccolingual plane at 6 μ m, and stained with hematoxylin and eosin. All histologic sections were coded and evaluated by an oral pathologist (T.H.M.), who was not familiar with the details of the study.

All radiographs were mounted in sequence for each animal and evaluated for signs of periapical pathosis in a blind study performed by a number of experienced endodontists and endodontic graduate students. Criteria included width of the periodontal ligament space, continuity of the lamina

| · | Matched pairs | Treatment history | Periapical radiographic observations | Periapical histologic evaluation |
|-------|---------------------------------------|---|--|--|
| Dog 1 | Right third premolar, mesial root | Control. Perforated apex. Saline Solution weekly. Observed 4 wk. | Two wk—widened PDL*space. Four wk—small periapical radiolucent area. | Widened PDL with mild fibro- sis across the foramen. Mild periapical inflammation ex- tending into root canal. Scat- tered PMNs, histocytes, lym- phocytes, and plasma cells. Bone remodeling (Fig. 3 A) |
| | Left third premolar, mesial root | Experimental. Perforated apex. R-595 weekly. Observed 4 wk. | Two wk—interruption of LD.† Four wk—small periapical radiolucent area and apical root resorption. | Periapical lesion with moderate inflammation, predominantly PMNs. Narrow extension of inflammation into mandibular canal. Bone remodeling and considerable root resorption (Fig 3, B). |
| | Right fourth premolar, mesial root | Experimental. Perforated apex. R-595 weekly. Observed 4 wk. | Two wk—small periapical radiolucent area and apical root resorption. Four wk—radiolucent area with continued root resorption. | Large periapical lesion. Peria- pical abscess with extension of inflammation into mandibular canal and purulent exudate into root canal. Scattered his- tiocytes, lymphocytes, and plas- ma cells. Bone remodeling and reservation (Fig 4, P) |
| | Left fourth premolar, mesial root | Control. Perforated apex. Saline solution weekly. Observed 4 wk. | Two wk—periapical os- teosclerosis. Four wk—osteosclerosis more pronounced. | Small periapical lesion. Scat- tered inflammatory cells, ede- ma, and fibrosis. Inflammatory tissue extending into root canal. Bone remodeling and root resorption (Fig 4). |
| Dog 2 | Left fourth premolar, mesial root | Control. Perforated apex. Saline solution weekly. Observed 4 wk. | Four wk—small periapical radiolucent area. | Periapical fibrosis and mixed infiltration of PMNs and chronic inflammatory cells. Edema. Acute inflammatory tissue and purulent exudate in root canal. Root resorption (Fig |
| | Right fourth premolar, mesial root | Experimental. Perforated apex. R-595 weekly. Observed 4 wk. | One vk—periapical radio- lucent area, diffuse. Four wk—larger diffuse radiolucent area and apical root resorption. | Large periapical abscess ex- tending into mandibular canal. Predominantly PMNs with some histiocytes and numerous plasma cells. Peripheral fibro- sis and bone remodeling. Root resorption (Fig 5,B). |
| | Left fourth premolar, distal root | Experimental. perforated apex. R-595 weekly. Observed 4 wks. | One wk—widened PDL space. Two wk—interruption of LD. Three wk—periapical ra- diolucent area, diffuse. | Periapical abscess extending into mandibular canal. Infil- trate predominantly PMNs, with some histiocytes, lympho- cytes, and plasma cells. Peri- pheral fibrosis and bone remodeling (Fig 6,B). |

Table • Radiographic and histologic results.

Table continues on facing page

| | Matched pairs | Treatment history | Periapical radiographic observations | Periapical histologic evaluation |
|--|---------------------------------------|---|---|---|
| | Right fourth premolar distal root | Control. Perforated apex. Saline solution weekly Observed 4 wk. | Four wkno change. | Fibrotic, widened PDL with edema and scattered PMNs. Narrow extension of inflam- mation into mandibular canal. Small amount of purulent exu- date in root canal. Minimal bone remodeling. Root resorp- tion (Fig 6,A). |
| | Left third premolar, mesial root | Control. Unperforated. Saline 0,2,3,4 wks. Observed 5 wks. | Five wk—no change. | Sections not available, because of technical problems. |
| | Right third premolar, mesial root | Experimental. Unperforated. R-595 0, 2, 3, 4, wks. Observed 5 wks. | Five wk—no change. | Mild PMN infiltrate. Bone remodeling (Fig 2). |
| | Left third premolar, dis- tal root | Control. Instrumented only. Unperforated. Observed 5 wks. | Five wks—no change. | Mild PMN infiltrate. Bone remodeling. |
| | Right third premolar, distal root | Control. Instrumented only. Unperforated. Observed 5 wks. | Four wk—widening PDL space. Five wk—interrupted LD. | Mild inflammatory cell infil- trate, predominantly PMNs. Bone remodeling. |

Table • Radiographic and histologic results (continued).

*Periodontal ligament.

†Lamina dura.

dura, root resorption, and obvious periapical radiolucent areas.

RESULTS

The animals were monitored daily and showed no signs of discomfort or distress. Clinical examination at weekly intervals showed no signs of inflammation, such as redness, swelling, or sinus tract stoma. No defects in the amalgam seals or fractures of teeth were detected.

The radiographic and histologic results are listed according to matched pairs in the Table. A high degree of radiographic standardization was achieved. In general, however, only a rough correlation between the radiographic and the histologic size of the lesions could be made. The radiographic lesions were mostly diffuse or poorly circumscribed, making radiographic quantification difficult. In the pairs with perforated apexes, the experimental roots containing endotoxin tended to show periapical radiographic changes sooner and to a more severe degree than did the matching control roots containing saline solution. The unperforated root containing endotoxin showed little or no radiographic changes, as did its matched root containing saline solution. The unperforated controls that had been instrumented only produced no radiographic changes over the duration of the study.

A relatively consistent histologic pattern developed in the periapical

tissues around the perforated apexes in response to the endotoxin; the bone defects were larger, and the inflammatory reaction more intense than those around the matched control roots containing saline solution (Fig 3-6). PMNs were the predominant cell type in the inflammatory infiltrations. The unperforated root containing endotoxin showed only minimal periapical inflammation. Histologic sections of its matched control containing saline solution were not available because of technical problems. The unperforated controls that had been instrumented only resulted in minimal to no inflammation at the end of the experimental period.



Fig 5—A, photomicrograph of perforated apex with saline solution; MC, mandibular canal; les, small periapical lesion with mixed inflammatory infiltrate and fibrosis; inf, acute inflammatory tissue extending into root canal with purulent exudate above. B, perforated apex with endotoxin; ab, large periapical abscess with inflammation extending into mandibular canal (MC); fib, peripheral fibrosis; pur, purulent exudate in root canal; res, root resorption. (H&E, orig mag $\times 31$).

DISCUSSION

Endotoxin molecules are lipopolysaccharides with large molecular weights of 1 to 2 million and have been chemically characterized as consisting of three principal regions. The lipid A region is linked by a trisaccharide of 2-keto-3-deoxyoctanoic acid (KDO) to a core polysaccharide, which is in turn linked to an O polysaccharide chain. The major antigen specificity of endotoxin molecules is related to the O polysaccharide region.34 The lipid A portion of the molecule is thought to be responsible for almost all of endotoxin's biological activities,35 including bone resorption.²⁷

The S minnesota R-595 organism is the product of the spontaneous morphological mutation of the parent wild or S strain (smooth colony form) to an R strain (rough colony form). R mutants have a block in cell wall biosynthesis of the O-specific repeating units of their endotoxin. The endotoxin extracted from the R-595 strain consists of lipid A and KDO without the core and O polysaccharides³⁵ and is, thus, about 70% lipid A.³⁶ The R-595 lipopolysaccharide has been shown to efficiently activate the classical pathway of complement⁶ and exhibit most other activities of complete endotoxin such as B-cell mitogenesis, lethal toxicity, Hageman factor activation, adjuvant effect, immunogenicity, and bone resorption.^{15,19,27,37,38}

Only minimal periapical response was seen associated with the roots in which apexes were not perforated, even the roots containing endotoxin. In light of the responses seen with perforated apexes, it seems reasonable to suspect that minimal endotoxin reached the periapex through the microscopic apical canal arborizations. Inflammation and bone destruction were seen to some degree with the perforated control roots containing saline solution. This would be expected, as perforation of apexes of dogs alone has been shown to produce an inflammatory reaction with resorption of cementum, dentin, and bone.39 The more intense infiltration of PMNs associated with the endotoxin-containing experimental roots is consistent with the ability of R-595 to activate complement and generate chemotactic factors. The larger periapical bone

lesions could be a reflection of the capacity of R-595 to affect tissue destructive mechanism, including bone resorption.

The experimental lesions did not resemble the typical chronic periapical lesion with its abundance of chronic inflammatory cells and dense fibrous encapsulation.⁴⁰ The weekly trauma of reinjection was probably a large factor in keeping the lesions acute as a similar but less intense histologic picture was seen with saline solution injections. Also, enhanced PMN mobilization has been shown to result from repeated peritoneal injections of the same endotoxin.⁴¹

An interesting observation with respect to endotoxin dose response was made by Greisman and Hornick⁴² when they injected humans intradermally with serial dilutions of purified bacterial endotoxins. Minimal inflammatory doses (about 10^{-3} mg/ml) elicited inflammatory responses at three to six hours with a mononuclear inflammatory cell population. As the dose was increased, the intensity of the early cellular inflammatory response increased and became progressively



Fig 6—A, photomicrograph of perforated apex with saline solution; inf, narrow extension of inflammation into mandibular canal (MC); res, root resorption; pdl, flattened fibrotic periodontal ligament with scattered PMNs and edema; pur, small amount of purulent exudate in root canal. B, perforated apex with endotoxin; ab, periapical abscess with extension of inflammation into mandibular canal (MC) and up lateral aspect of root (inf); fib, periapical fibrosis (H&E, orig mag $\times 31$).

more polymorphonuclear in nature until PMNs predominated at concentrations one-hundred-fold above the minimal inflammatory dose. It has also been shown that higher concentrations (around 10 μ g/ml) of several endotoxin preparations have resulted in less osteoclastic bone resorption in vitro than have lower concentrations (1 to 3 μ g/ml), suggesting a toxic effect at the higher dose levels.26,28 High concentrations of endotoxin can also inhibit activation of the Hageman factor.15 Conceivably, smaller doses of endotoxin than were used in this study could result in a histologic picture that resembles more the typical chronic periapical lesion.

Species variation in susceptibility to the many effects of endotoxin and to the route of administration can also have a bearing on the results seen with endotoxin use.⁴³ Thomas⁴⁴ suggests that dogs and cats are less susceptible to endotoxin than are mice, rats, guinea pigs, and rabbits. Wolff⁴⁵ considers humans to be the most sensitive to the biological effects of endotoxin. As with responses to other categories of antigens, decided differences in the immune response of different animal species to endotoxins have been reported.²⁰ Species differences in complement activation have also been shown.^{46,47}

The dog model offered multiple matched pairs of experimental sites and permitted standardized radiographic techniques. However, because the main canal is not continuous to the root apex, mechanical perforation of the apexes, along with the attendant periapical trauma, was necessary to deliver the endotoxin to the periapexes. Also, because of the large diameter of the mandibular canal and its proximity to the root apexes, in some areas, only a few trabeculae of bone separate the two (Fig 3,B). Periapical lesions tended to extend readily into the canal, making it more difficult to evaluate the extent of destruction of the periapical bone.

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