# Endotoxic activity in periapical lesions

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Thirty tissue samples were examined histologically and classified as being inflamed (apical granulomas) or noninflamed (scars or noninflamed cysts). The samples were then homogenized in pyrogen-free water and treated to remove interfering substances. The presence of endotoxin was then determined by means of the Limulus assay; 75 percent of the inflamed tissues were positive for endotoxin, while only 20 percent of the noninflamed tissues contained endotoxin. The presence of endotoxin was thus highly correlated (p = 0.015) with the presence of inflammation in these tissues.

The pathogenesis of the chronic periapical granuloma is not well understood. Although the presence of oral bacteria in these tissues could account for much of the pathosis seen, several studies have shown these lesions to be sterile.<sup>1-3</sup> More recently, attention has been directed toward the possibility that host immunologic mechanisms mediate much of the tissue destruction seen.<sup>4-3</sup> Indeed, several recent studies have demonstrated the presence of various immunoglobulins and complement components in these tissues.<sup>6-9</sup>

A possible explanation for the pathogenesis of the chronic periapical granuloma is that bacterial products originating in septic root canals diffuse into the apical tissues, thus eliciting host responses. Since gram-negative bacteria are frequent isolates of infected root canals,<sup>10-12</sup> bacterial endotoxin would be a likely candidate for one such substance. The presence of endotoxic activity has recently been demonstrated in root canals of teeth with necrotic pulps,<sup>13, 14</sup> but we are not aware of any published reports demonstrating the presence of endotoxin in apical tissues. In this article we report the presence of endotoxin in 75 percent of apical lesions with the histopathologic characteristics of chronic granulomas. Endotoxic activity was found in only 20 percent of the noninflamed oral tissues that we examined.

### MATERIALS AND METHODS Source of tissues

Apical lesions were obtained from teeth without obvious periodontal involvement. The tissues were taken at the time that therapeutic apicectomies were performed. In most cases, approximately one half of the lesion was placed in formalin fixative and submitted to an oral pathologist for histologic evaluation. The remainder of the tissue was placed in sterile plastic 1.5 ml. conical centrifuge tubes and immediately frozen; the tissues were kept frozen until the time of analysis in the laboratory.

In those cases in which no pathology report was available, frozen sections of the tissues were cut in our laboratory and stained with hematoxylin and eosin. The tissues were then examined microscopically and classified as either granuloma, apical scar, or cyst according to the following criteria: Granulomas were characterized by a dense round-cell infiltrate (with or without a cystic epithelium); apical scars were characterized by the presence of dense connective tissue without appreciable inflammation; and cysts were characterized by the absence of inflammation and the presence of an epithelial lining. Photomicrographs of typical tissues graded as granuloma and apical scar are shown in Figs. 1 and 2, while an outline of the experimental protocol is given in Fig. 3.

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Fig. 1. Photomicrograph of a typical granulomatous lesion. Note the heavy lymphocytic infiltrate characteristic of chronic inflammation. (Approximate magnification,  $\times 250$ .)

In addition to the apical lesions, we obtained a piece of noninflamed gingival tissue to serve as another control. This tissue was taken during a therapeutic gingivectomy and was otherwise treated the same as the apical tissues. The absence of appreciable inflammation was confirmed histologically.

#### Preparation of tissue homogenates

All laboratory procedures were carried out with sterile pyrogen-free (endotoxin-free) instruments, glassware, and reagents. Pyrogen-free disposable materials were used wherever possible. Reusable instruments and glassware were rendered pyrogenfree by baking at 180° C. for at least 4 hours.

At the time of analysis the tissues were thawed, weighed, and placed in glass microhomogenizers. They were then homogenized in the presence of 100  $\mu$ l of sterile, pyrogen-free water per milligram of tissue. The homogenates were transferred to centrifuge tubes and centrifuged at 1,400 × g for 20 minutes to pellet the cellular debris. The supernatants were then treated to remove interfering substances and assayed for the presence of endotoxin.

#### **Removal of interfering substances**

We used the Limulus amebocyte lysate (LAL) assay to detect endotoxin. Although this assay was once thought to be quite specific for the detection of endotoxin. in fact, a number of substances can interfere with the assay by giving both false positive and false negative results. These substances include certain plasma proteins, polynucleotides, and the peptidoglycan from gram-positive bacterial cell walls.<sup>15-18</sup> We therefore performed control experiments to ensure that these substances were not interfering with our data. (Because of the limited amounts of homogenate available from each tissue specimen, we were able to perform a complete set of controls on only ten of the thirty specimens analyzed.)

In early experiments, the "inhibition controls" (see below) indicated the presence of a substance which gave a false negative result. We discovered that this substance was DNA; therefore, every sample was incubated at 37° C. for 1 hour with 20 units of pyrogen-free DNAse (deoxyribonuclease, Worthington Division of Millipore Corp., Freehold, N.J.) per 100  $\mu$ l of sample.



**Fig. 2.** Photomicrograph of a typical apical scar. Note the density of the connective tissue and absence of inflammation. (Approximate magnification,  $\times 250$ .)

To rule out the possibility that gram-positive peptidoglycan was responsible for giving a false positive test result, portions of ten samples were treated with 180 units of lysozyme ( $3 \times$  recrystallized, Calbiochem-Behring Corp., La Jolla, Calif.) per 100  $\mu$ l of sample. This treatment was performed concomitantly with the DNAse treatment on these ten samples (Fig. 3).

Those samples treated with the lysozyme were further treated to degrade noncollagenous proteins. This was done by incubating the samples for an additional hour at 37° C. with 2,000 units of trypsin (pancreatic, A grade, Calbiochem-Behring Corp., La Jolla, Calif.) per 100  $\mu$ l of sample.

In order to denature any other interfering substances which might be present, all samples were boiled for 20 minutes, (Endotoxin itself is not affected by boiling.) These various experimental manipulations are diagrammed in Fig. 3.

## Limulus amebocyte lysate assay

The principle of the LAL assay is as follows: A lysate prepared from the circulating amebocytes of the horseshoe crab (*Limulus polyphemus*) is incubated with the material to be tested. The presence of

endotoxin causes the amebocyte lysate to clot, which results in the formation of a turbid, adherent mass at the bottom of the test tube.<sup>19</sup> (All reagents used for this assay were purchased from Microbiological Associates, Walkersville, Md.)

The assay was performed by reconstituting a premeasured lyophilized portion of Limulus lysate with 200  $\mu$ l of sample which had been treated to remove interfering substances (see above). In addition, positive, negative, and "inhibition" controls were also prepared. The positive control consisted of lysate reconstituted with a solution known to contain 0.2 ng of purified endotoxin from *Escherichia coli* (strain 0111:B4), while the negative control consisted of lysate reconstituted with endotoxin-free water. The purpose of the inhibition control was to detect the presence of substances which would inhibit clot formation; it consisted of reconstituting the lysate with the experimental sample in the presence of bona fide endotoxin.

The samples and controls were incubated at 37° C. for 1 hour. Following incubation, the tubes were inverted. Test results were considered to be positive (endotoxin present) when the sample, positive control, and inhibition control each had a

turbid, adherent clot at the bottom of the tube and when the negative control remained unclotted. Test results were considered to be negative when the sample and negative control were unclotted and the positive and inhibition controls were clotted. Any other combinations were scored as "inconclusive" and are not reported.

## RESULTS

As described above, one third of the samples were treated both with DNAse only and with DNAse, lysozyme, and trypsin prior to the LAL assay (Fig. 3). The results of LAL testing following these two modes of sample preparation were identical (Table I), indicating that no substances which could have given a false positive test result were removed by the incubations with lysozyme and trypsin. In this first group of samples, 71 percent of the granulomas showed a positive LAL reaction, while 33 percent of tissues diagnosed as apical scars were positive for endotoxin.

Because the results for the two modes of sample preparation were the same, the remaining twenty samples were treated with DNAse only. In this group, 77 percent of the granulomas were positive for endotoxin, while only 17 percent of the noninflamed tissues gave a positive LAL reaction (Table II).

When the data from these two tables are combined, we find that 75 percent of the lesions diagnosed as granulomas were positive for endotoxin, while only 20 percent of the control tissues gave a positive LAL test response (Table III). The presence of endotoxin is thus highly correlated with the presence of inflammation (p = 0.015 by chi-square analysis).

# DISCUSSION

Although the LAL assay is a very sensitive indicator of the presence of bacterial endotoxin, there are a variety of other substances which can induce or prevent clotting of the lysate. Among these substances are thrombin, thromboplastin, and polynucleotides<sup>15</sup>; peptidoglycan from gram-positive organisms<sup>15</sup>; other blood components<sup>17</sup>; and compounds found in inflammatory exudates.<sup>18</sup>

The LAL assay is generally not as sensitive to the presence of these substances as it is to the presence of endotoxin. Furthermore, many of these substances can be enzymatically degraded<sup>16, 20</sup> or denatured by boiling,<sup>17</sup> thus eliminating their effect on the assay.

In our study, we used a combination of enzymatic degradation and heat denaturation to ensure the specificity of the LAL test. For example, after we



Fig. 3. Flow chart of experimental manipulations. All thirty tissue samples were divided and a piece was used for histologic evaluation. The remainder of each tissue sample was homogenized in pyrogen-free water. Ten homogenates were divided into two groups at this point, with one half of each of these ten homogenates being treated with DNAse and boiled prior to the LAL assay. The other half of the homogenate was treated with DNAse, lysozyme, trypsin, and finally boiled prior to the LAL assay. The other twenty samples were treated with DNAse and boiled prior to the LAL assay. (See text for details.)

determined that the presence of DNA in the sample homogenate inhibited gelation of the lysate (thus giving a false negative result), all subsequent samples were incubated with DNAse to eliminate this problem. All samples were also boiled to denature other possible interfering substances.

Ten samples were treated both with the DNAse/ boiling protocol and with a protocol that added incubations with lysozyme and trypsin. The lysozyme served to degrade any gram-positive cell wall peptidoglycan that might have been present, while the trypsin degraded any proteins that might have interfered with the assay. In all cases, the results of the DNAse/boiling protocol and the "complete" sample preparation protocol were identical. This indicates that interfering substances either were not present in concentrations which would affect the LAL assay or were inactivated by the DNAse/ boiling treatment.

If the positive LAL test responses were due to the

		LAL test results		
Patient	Tissue	DNAse only	DNAse, lysozyme, trypsin	
N.K.	Scar			
V.M.	Granuloma			
N.S.	Granuloma			
J.S.	Granuloma	+	+	
P.S.	Scar	-	_	
F.U.	Granuloma	+	+	
H.B.	Scar	+	+	
B.B.	Granuloma	+	+	
A.A.	Granuloma	+	+	
Z.B.	Granuloma	+	+	

Table I. Effects of pretreatment on Limulus assay

Table II. Limulus assay results using DNAse only

Patient	Tissue	LAL test result
Т.В.	Cyst	
R.V.	NGT*	
G.M.	Granuloma	+
S.T.	Granuloma	+
D.C.	Granuloma	+
M.C.	Granuloma	+
R.H.	Cyst	
N.F.	Granuloma	+
<b>B</b> .G.	Scar	-
Т.В.	Granuloma	+
D.B.	Granuloma	+
L.C.	Granuloma	
<b>R.S</b> .	Scar	+
K.B.	Granuloma	_
D.C.	Granuloma	+
S.H.	Granuloma	_
H.F.	Scar	-
D.J.	Granuloma	+
N.S.	Cyst	-
S.C.	Granuloma	+

\*Noninflamed gingival tissue.

presence of lysate activators other than endotoxin, one would expect these substances to be present in homogenates from all of the granulomas. Yet 25 percent of the granulomas (all of which were chronically inflamed by histologic criteria) did not yield positive LAL test results. This is additional evidence that the LAL assay was specifically detecting endotoxin under the conditions we used.

The presence of endotoxin in the lesions was found to be highly correlated with the presence of inflammation; that is, the granulomatous lesions were much more likely to contain endotoxin than were the noninflamed tissues. (The correlation was found to be significant at p = 0.015 when chisquare statistics were used.) The high degree of correlation rules out contamination from the operative field as a significant source of endotoxin. If such

Table III.	Summary	of	data	in	Tables	I	and	IJ	L
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	LAL test re	sults
Tissue	+	
Granuloma	15	5
Others	2	8

contamination were significant, one would expect a higher percentage of the noninflamed tissues to have been positive for endotoxin, since it is difficult to imagine that the granulomatous lesions would have been more prone to contamination than the control tissues.

The demonstration of endotoxin in apical granulomas has significant implications for an understanding of the etiology of these lesions. While some may argue that these lesions are not sterile, the frequency with which studies have failed to find viable organisms in apical tissues<sup>1-3</sup> indicates that they are not grossly septic. If there are not great numbers of bacteria in these lesions, what accounts for their pathogenesis?

One possibility is that bacterial products escape into the apical tissues from septic root canals. These substances may have intrinsically deleterious effects on the connective tissue cells surrounding the apex of the tooth; they are undoubtedly antigenic and would trigger host immunologic responses which, in turn, can mediate inflammation and tissue destruction.<sup>4, 5</sup>

Endotoxin from gram-negative bacteria is a substance which is directly toxic for a variety of cell types, including fibroblasts,<sup>21</sup> and can activate the complement system via the alternate pathway.<sup>22</sup> Complement activation leads to the generation of anaphylatoxic and chemotactic peptides<sup>23</sup> which are potent mediators of inflammation and which are important in the pathogenesis of the Arthus reac-

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tion. Moreover, relatively high concentrations of endotoxin have recently been found in the canals of teeth with necrotic pulps.<sup>13, 14</sup>

Our results indicate that a bacterial product (endotoxin) which is present in the root canals of teeth with necrotic pulps is, in fact, present in the periapical areas of teeth with apical granulomas. The presence of such products in periapical tissues can account for the occurrence of apical disease, even if no viable bacteria are present.

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