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Induction of Nonspecific Tolerance to Endotoxins Reduces the Alveolar Bone Resorption in Ligature-Treated Rats

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Previous experimental data from various laboratories indicate that endotoxin of gram-negative oral microorganisms might be one of the most important bacterial products involved in bone resorption during periodontitis. Immunologically nonspecific tolerance to endotoxins in rats was induced by repeated application of *Serratia marcescens* trichloroacetic acid-extracted endotoxin. Silk ligature was placed on the second maxillary molar of the endotoxin-tolerant rats as well as of control rats in which tolerance to endotoxin had not been induced. The animals were sacrificed 8 days later. The rats showed no specific immune response to the tolerance-inducing endotoxin as measured by passive hemagglutination and by the lymphoblast assays, but we found that bone resorption was significantly reduced in the endotoxin-tolerant rats as compared with ligature-treated animals in which tolerance to endotoxin had not been induced.

Tolerance to the various effects of endotoxins can be induced by their repeated applications. This tolerance is manifested by unresponsiveness to pyrogenic or to lethal dose challenges, and it is immunologically nonspecific, since endotoxin from any gram-negative bacterium can induce tolerance to other endotoxins which are serologically unrelated to it.

Tolerance to experimental infection with lethal doses of viable pathogenic microorganisms can also be elicited by endotoxins. This tolerance, often called enhanced nonspecific resistance (NSR), is also immunologically nonspecific since any endotoxin will induce such NSR to gram-negative organisms or to some gram-positive organisms or even to viral challenges in spite of the lack of cross-reacting immunodeterminants in the tolerance-inducing and the challenging preparations. The major difference between the tolerance to endotoxin and enhanced NSR is that while NSR can be enhanced by a single low dose (0.01 to 0.1 μg per mouse), the induction of endotoxin tolerance requires repeated applications of greater endotoxin quantities, such as 5 μg or more per mouse.

Results from our laboratories as well as from several other institutions showed that endotoxin is most likely involved in the pathogenesis of periodontitis (3, 5-7, 14). We assumed, therefore, that induction of tolerance to the pathogenic effects of endotoxins or to the invasion of viable microorganisms (NSR) or both will reduce alveolar bone resorption in experimental models of periodontitis.

The model we are using involves ligature application on rat teeth. Silk ligature placed on the maxillary molar induces slight injury and a visible inflammation of the gingival tissues (4, 16). Bacterial accumulation on and around the ligature has been reported (4, 8, 16). The bacterial by-products, among them endotoxin from gram-negative microorganisms, are released, and they penetrate the gingival tissues (12, 18). Bone resorption develops in 8 to 12 days (4, 8, 13) and can be greatly accelerated by immunosuppression (16, 17). Elimination of microorganisms by antibiotic therapy prevents inflammation and subsequent bone resorption activity (16, 19).

The ligature-induced periodontitis model was utilized to study the effect of endotoxin tolerance on the alveolar bone resportion. The results of these experiments are reported here.

MATERIALS AND METHODS

Animals. Sprague-Dawley rats weighing 200 to 300 g were purchased from Charles River Breeding Laboratories, Inc., Wilmington, Mass., and maintained on a regular diet during the experiments.

Endotoxins. *Serratia marcescens* O8 bacteria were extracted by the trichloroacetic acid procedure of Boivin as modified by Nowotny (9) and purified by precipitation with ethanol and by sedimentation in an ultracentrifuge at 100,000 × *g* for 3 h. *Salmonella minnesota* 1114 bacteria were extracted and purified by the phenol-water procedure of Westphal and Lüderitz (20).

Mitogenic response. Cellular responses to *S. marcescens* O8 endotoxin as well as to concanavalin A were

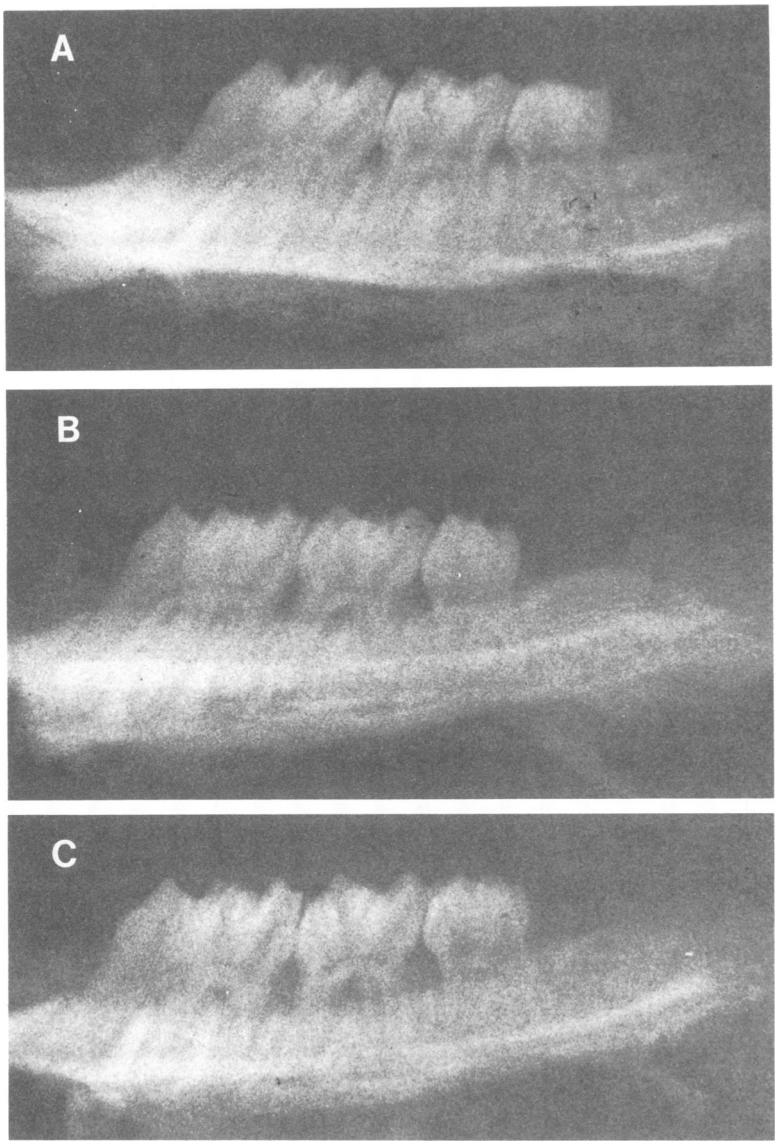

FIG. 1. Radiograph of rat maxilla. (A) Normal, (B) effect of tolerance induction and ligature, (C) ligature only.

determined as previously described (9). Spleen cells of individual animals adjusted to 2×10^6 cells in a total volume of 1 ml in RPMI medium with 10% fetal calf serum were incubated in the presence of 10 μg of endotoxin or 5 μg of concanavalin A for 68 h at 37°C in 5% CO_2. Each triplicate cell culture was pulsed with 1 μCi of [^{3}H]thymidine (specific activity, 53 Ci/mmol; New England Nuclear Corp., Boston, Mass.) for an additional 4 h. At this time, the cells were harvested, and their radioactivity was determined by counting the samples for 1 min in a liquid scintillation counter. The results were expressed as counts per minute of the sample divided by the counts per minute of the saline controls. This quotient is presented as the stimulation index.

Anti-*S. marcescens* antibody determination. Titers of serum antibody directed against *S. marcescens* O8 were determined by passive hemagglutination. To 50 μl of heat-inactivated rat serum samples serially diluted 1:2 with phosphate-buffered saline in microtiter plates was added 50 μl of a 2% suspension of sheep erythrocytes coated with *S. marcescens* O8 endotoxin. Sheep erythrocytes were coated with endotoxin by the standard Boyden procedure as described by Nowotny (9). After 4 h of incubation at room temperature, the results of passive agglutination were expressed as the reciprocal of the $\log_2$ of the highest dilution of serum which still exhibited visible agglutination.

Application of ligature. Rats were anesthetized by

TABLE 1. Demonstration of endotoxin tolerance in rats by the lethality test

Group	Treatment	% Survival (72 h)
I	Three injections of 8 μg of LPS[a]	80
II	One injection of 8 μg of LPS	60
III	One injection of 50 μg of LPS	100
IV[b]	One injection of 50 μg of LPS	80
V	Control saline	0

[a] *S. marcescens* LPS was used for this study. The 50% lethal dose is 35 mg/kg.

[b] Induction of endotoxin tolerance was with *S. marcescens* LPS, but 50 mg of *S. minnesota* LPS per kg was given in the lethality test 24 h after the last tolerance-inducing injection.

injecting 0.03 ml of Innovar-vet. Sterilized 000 silk ligature (nonabsorbable surgical suture A-54 Ethicon; Ethicon Inc., Somerville, N.J.) was placed around the cervix of the second molar. The ligature was knotted on the palatinal side of the tooth.

Measurement of the bone resorption. The maxillary jaws were dissected, boiled for 2 h in water, cleansed of any soft tissue, and left overnight in 3% peroxide solution.

To determine the alveolar bone loss in the buccal and interproximal area, direct microscope measurements of the prepared specimens and of radiographic films were carried out as follows. Specimens were placed on a dissecting microscope, and the distance from the cemento enamel junction to the most coronal part of the alveolar crest was recorded. These measurements were made with a calibrated grid with a density of 0.01 mm placed in the ocular of a dissecting microscope. Three measurements of mesial, midbuccal, and distal were registered.

In addition, radiographic examinations were also conducted to record the interproximal bone loss. The maxillary jaws were placed in front of film and exposed at 60 KUP, 10 mA, and 10 pulses using the long cones. The film was processed, and Fig. 1 shows a radiograph obtained by this procedure. Measurements were done on an X-ray viewer with a magnifying glass with built-in millimeter rules. The density of the ruler was 0.02 mm. All specimens and radiographs were coded, and measurements were done by three examiners in a blind fashion.

Induction of endotoxin tolerance. Various schedules for induction of tolerance to endotoxin were used. *S. marcescens* endotoxin was injected intraperitoneally either once or repeatedly in 8- or 50-μg quantities (Table 1).

The effectiveness of the tolerance induction was determined by two procedures: (i) by rectal temperature measurements and (ii) by challenge with lethal doses of endotoxin. Normal rats and mice respond with a drop in body temperature to the first injection of endotoxins, but rats with induced tolerance respond with either no change or a slight elevation of body temperature. Lethal challenge with the tolerance-inducing endotoxins or with serologically unrelated endotoxins is a more reliable way to determine the effectiveness of induction of endotoxin tolerance. The procedures for both assays were described previously (9).

RESULTS

Induction of endotoxin tolerance. Elevation of the body temperature of the rats (indicating tolerance) could be seen after three intraperitoneal injections of 8 μg of *S. marcescens* endotoxin per animal repeated daily. Figure 2 shows the results obtained by this schedule.

The effect of various schedules for induction of endotoxin tolerance was also tested by the response of rats to lethal challenges with either the tolerance-inducing *S. marcescens* endotoxin or *S. minnesota* lipopolysaccharide (LPS). The results summarized in Table 1 show that good tolerance could be achieved by several schedules.

Antibody levels. Animals were sacrificed 8 days after the last injection. The sera were collected, and their antibody titers were determined individually by passive hemagglutination. *S. marcescens* endotoxin-coated sheep erythrocytes were used in these assays. Anti-*S. marces-*

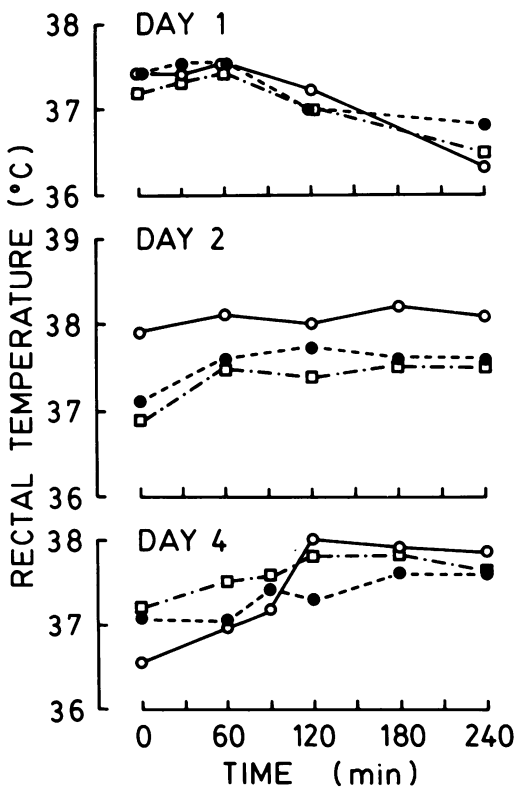

FIG. 2. Rectal temperature measurements of three Sprague-Dawley rats (○, ●, □) after the injection of 25 μg of LPS. The effects of the first (day 1), second (day 2), and fourth (day 4) injections are shown.

TABLE 2. Lymphoblast response in ligature-treated rats with induced endotoxin tolerance

Group	Treatment	Stimulation index with:	
		10 μg of endotoxin	5 μg of concanavalin A
I	Three injections of 8 μg of *S. marcescens* LPS	1.33	4.31
II	One injection of 8 μg of *S. marcescens* LPS	1.93	6.48
III	One injection of 50 μg of *S. marcescens* LPS	1.63	5.45
IV	No LPS	1.33	7.26

TABLE 3. Alveolar bone loss in ligature-treated rats after various tolerance induction treatments (gross measurement)

Group	Treatment	Bone loss (mm)		
		Distal	Midbuccal	Mesial
I[a]	Three injections of 8 μg of *S. marcescens* LPS before ligature placement	0.56 ± 0.2[b]	0.56 ± 0.19[b]	0.64 ± 0.3[c]
II	One injection of 8 μg of *S. marcescens* LPS before ligature placement	0.94 ± 0.4[c]	0.94 ± 0.5[c]	0.78 ± 0.4[c]
III	One injection of 50 μg of *S. marcescens* LPS	0.30 ± 0.08[b]	0.33 ± 0.1[b]	0.39 ± 0.08[b]
IV	Ligature	1.64 ± 0.1[c]	1.18 ± 0.3[c]	1.48 ± 0.3[c]
	Control	0.26 ± 0.08[b]	0.25 ± 0.09[b]	0.30 ± 0.1[b]

[a] Ten rats were used in each group.
[b] $P < 0.02$ compared with ligature group IV.
[c] $P < 0.02$ compared with the control site (no ligature).

cens O8 immunoglobulins were not detectable in any one of the sera of the rats with tolerance induced by three 8-μg doses. Very low levels of antibodies were detected in a few sera if a single 50-μg dose was given.

Lymphoblast assay. The results of these measurements are expressed as stimulation indices in Table 2. Accordingly none of the tolerance-inducing schedules sensitized the rats.

Bone resorption. Table 3 summarizes these results. Almost complete prevention of the alveolar bone loss could be achieved by three 8-μg injections and one 50-μg injection of *S. marcescens* endotoxin. The same schedules resulted in induction of endotoxin tolerance and no significant increase in the antibody levels. The least effective of the schedules was the single treatment of 8 μg per rat. The total number of rats per group is also indicated in Table 3.

Table 4 shows the effect of tolerance induction on the bone loss determined by radiography.

TABLE 4. Alveolar bone loss in ligature-treated rats after tolerance induction (X-ray measurement)

Group	Treatment	Bone loss (mm)	
		Distal	Mesial
I	Three injections of 8 μg of *S. marcescens* LPS before ligature placement	0.52 ± 0.1	0.59 ± 0.1
IV	Ligature	1.20 ± 0.2	1.09 ± 0.3

Figure 3 shows a photograph of defleshed rat maxilla. Figure 3A shows normal rat teeth. Figure 3B shows the effect of tolerance induction on the ligature-caused bone resorption, which can be compared with the effect of the ligature on the alveolar bone of rats in which tolerance to endotoxin was not induced, shown in Fig. 3C.

DISCUSSION

Results of this study indicate that bone destruction in this model of periodontitis could be greatly reduced if the animals were systemically treated to induce tolerance to endotoxin before ligature placement.

There are several mechanisms which one should consider in searching for a possible explanation of the observed phenomenon. As described in the introduction, tolerance to endotoxic reactions and tolerance to viable bacteria (NSR) are well-known consequences of endotoxin injections. Endotoxins are also known to be highly active immunopotentiators. Finally, endotoxins are immunogenic and antiendotoxin antibodies can neutralize some of the toxic effects of endotoxins (11).

Immunopotentiation may help the animals to develop a protective antibody level to those bacteria which accumulated on the ligature and penetrated the gingival epithelium. Since the

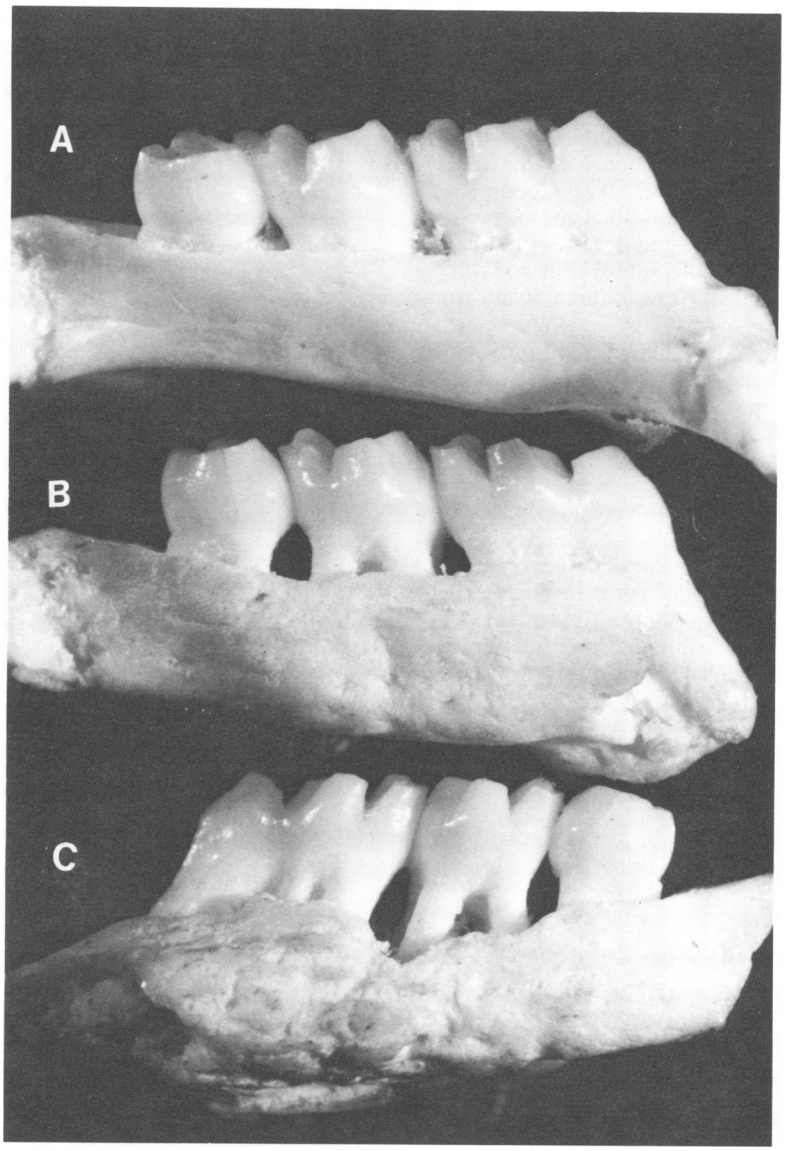

FIG. 3. Defleshed rat maxilla. (A) Normal, (B) effect of tolerance induction and ligature, (C) ligature only.

ligature is on for 8 days, there is sufficient time for antibody production. If such an antibody level is developed, the immunoglobulins should be specific to the various immunogens of the microorganisms, but they can be detected only by using isolated strains of the microflora. We are in the process of isolating and identifying the major strains which colonized the ligature (16), and we intend to use these for the detection of local antibody production.

One of the least probable mechanisms appears to be the enhancement of NSR. It is clear that single low-dose endotoxin injection enhances the NSR, most probably via activation of the reticuloendothelial system (1, 2, 10, 15). The fact that a single 8-μg injection was the least effective in reducing the bone loss makes it unlikely that enhanced NSR would be responsible for the here-observed protective effect.

We may also conclude with considerable certainty that the protection induced by repeated injections of *S. marcescens* O8 endotoxin is not a consequence of specific immune reaction to this bacterial strain. First of all, none of the schedules resulted in significant levels of anti-*S. marcescens* O8 immunoglobulins. Furthermore, even if there would be an effective local immune defense developing to *Serratia* strains, it would

be probably without beneficial effects to the host, since *Serratia* strains were not among those that we could isolate from the ligature.

We demonstrated that the rats were tolerant to lethal endotoxin challenge. They were induced to endotoxin tolerance with *S. marcescens* O8 and challenged with *S. minnesota* 1114 endotoxin. We can assume that they were also renderd tolerant to the endotoxins of the bacteria which colonized the suture, but convincing proof of this can only be offered if all gram-negative bacteria are isolated and their endotoxins used in lethal dose challenges to test tolerance to these isolates.

In conclusion, the most likely mechanism responsible for the here-described effect is the induction of immunologically nonspecific tolerance to endotoxin. This statement does not intend to exclude the involvement of other protective mechanisms, some of which were discussed above. Our present efforts are aimed to optimize the protection, test it in other experimental models of periodontitis, and gain a better insight into its possible mechanisms both at cellular and molecular levels.

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