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Biological Activities of *Eikenella corrodens* Outer Membrane and Lipopolysaccharide

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Highly purified preparations of the outer membrane and lipopolysaccharide (LPS) of *Eikenella corrodens* strain ATCC 23834 and the outer membrane fraction (OMF) of strain 470 were tested in in vitro biological assays. The OMFs of both strains were found to be mitogenic for BDF and C3H/HeJ murine splenocytes. The *E. corrodens* LPS was mitogenic for BDF spleen cells; however, doses of LPS as high as 50 μg/ml failed to stimulate C3H/HeJ cells. When incubated with T-lymphocyte-depleted C3H/HeJ splenocytes, the strain 23834 OMF demonstrated significant mitogenic activity, indicating that the OMF is a B-cell mitogen by a mechanism other than that elicited by conventional LPS. The *E. corrodens* 23834 OMF and LPS were stimulators of bone resorption when tested in organ cultures of fetal rat long bones. In contrast, the strain 470 OMF was only weakly stimulatory. Both OMFs and LPSs demonstrated "endotoxic" activity, since as little as 0.062 μg of *E. corrodens* LPS and 0.015 μg of the OMFs induced gelation in the *Limulus* amebocyte clotting assay. Thus, despite having a "nonclassical" LPS biochemistry, the *E. corrodens* LPS elicits classical endotoxic activities. These results also indicate that the surface structures of *E. corrodens* have significant biological activities as measured in vitro. The expression of such activities in vivo may play an important role in the pathogenesis of periodontitis as well as other *E. corrodens* infections.

It is now well recognized that inflammatory periodontal disease is an infectious process, and the presence of specific bacteria in the gingival crevice is both essential and probably central to the tissue destruction encountered (17, 20, 21). *Eikenella corrodens* is one of several species of gram-negative bacteria routinely isolated from subgingival plaque and periodontal pockets (20, 24). The genus *Eikenella* appears to be associated with cases of advanced periodontitis that are characterized by minimal clinical inflammation, but severe bone loss (24). In such cases, despite the apparent presence of relatively few microorganisms at the diseased site, a relatively rapid destruction of the tooth-supporting tissues occurs. This situation, according to Tanner and coworkers (24), may indicate that the etiological agent is highly virulent; *E. corrodens* is recovered in high numbers relative to the other microorganisms in these sites and may thus be a major contributor to the disease process. The periodontopathic nature of *E. corrodens* in animal model systems has been firmly established by studies showing that oral colonization of *E. corrodens* in gnotobiotic rats results in severe periodontal attachment loss within a matter of weeks (9, 11). Thus the link of *E. corrodens* to periodontitis is circumstantially strong.

The tissue destruction encountered in periodontal disease may be due to direct cytotoxic effects of the periodontopathic bacteria, activation of the host's immune response by the bacterial components, or both (14). A number of bacterial components or cell products have either been reported to be or have the potential to be directly cytotoxic (4, 8, 18, 22). *Actinobacillus actinomycetemcomitans*, which has also been implicated in juvenile periodontitis, produces a soluble, heat-labile leukotoxin that destroys human gingival polymorphonuclear leukocytes (PMNs) (23). In addition, Robertson et al. (18) have reported that periodontitis-associated bacteria, including several species of *Bacteroides* and certain strains of *A. actinomycetemcomitans*, produce a collagenase that could be important in periodontal connective tissue destruction. Peptidoglycans (Holt, unpublished data) are potent mediators of macrophage and fibroblast activation. Another cellular component of gram-negative periodontopathogens that may have several effects on various periodontal tissues is lipopolysaccharide (LPS). It not only is a potent stimulator of bone resorption (7), but also weakens lysosomal membranes, releasing lytic enzymes into the extracellular environment (10), as well as having direct cytotoxic effects on cultured fibroblasts (13) and peritoneal macrophages (K. A. Barker and S. C. Holt, in press).

Since periodontal tissues are in direct contact with a variety of gram-negative bacteria of significant periodontal potential, including *E. corrodens*, the surface components of these bacteria likely play an important initial and possible active role in the periodontal disease process.

This report describes the activities of purified *E. corrodens* surface structures, i.e., LPS and outer membrane fractions (OMFs), in several in vitro systems to define the possible role of this microorganism in the destruction of the supporting dental tissues in the course of periodontal disease.

MATERIALS AND METHODS

Organism. *E. corrodens* strains ATCC 23834 and strain 470 from S. S. Socransky, Forsyth Dental Center, were used in this study. Both cultures were grown as described elsewhere (A. Progulske, personal communication) in BY broth.

Isolation of cellular fractions. The OMF from strain 23834 and strain 470 as well as the LPS from strain 23834 were isolated and purified as previously described (15).

Measurement of bone resorption stimulation. The ability of outer membrane and LPS preparations to stimulate resorp-

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tion was measured in organ cultures of fetal rat long bones as previously described (16, 25). Pregnant rats were injected subcutaneously with 200 μCi of ^{45}Ca on day 18 of gestation. After 24 h, the calcified shafts of the fetal radii and ulnae were removed by microdissection and explanted to culture in BGJ medium (16) supplemented with bovine serum albumin (1 mg/ml). The cultures were incubated at 37°C in an atmosphere of 5% CO_2 in air. After 24 h of incubation in control medium to reduce the amount of readily exchangeable ^{45}Ca, the bones were transferred to fresh medium with or without the test substance and cultured for 48 h. After measurement of bone and medium ^{45}Ca content by liquid scintillation counting, the release of ^{45}Ca from the bone was calculated as the percentage of the total amount originally present. These data were then expressed as the ratio of ^{45}Ca released from treated bones relative to untreated control bones (T/C). A mean T/C ratio significantly greater than 1.0 was taken to indicate stimulation of resorption by the test substance.

Measurement of *Limulus* amebocyte clotting activation. The gelation of *Limulus* amebocyte lysate by *E. corrodens* LPS and outer membrane preparations was measured as described by Haemachem, Inc. (St. Louis, Mo.) with Haemachem reagents (1). Only pyrogen-free plastic and glassware was used in the assay procedure. Reference LPS (from *Escherichia coli* O55:B5), *E. corrodens* cellular fractions, and the limusate reagent were reconstituted in pyrogen-free water. Dilutions of test and control samples (*E. coli* LPS and pyrogen-free water) were incubated with the amebocyte lysate at 37°C for 60 min. The presence or absence of a gel after incubation was determined by inverting the tubes once; any tube that contained a gel that did not adhere to the bottom of its tube was scored as negative.

Measurement of mitogenic activity. *E. corrodens* cell fractions were assayed for mitogenic activity by a method similar to that of Chen et al. (5). Briefly, murine spleen cells from strains of C3H/HeJ and BDF mice were cultured at a concentration of 4×10^5 cells per microtiter well containing 0.2 ml of RPMI 1640 medium supplemented with sodium pyruvate, L-glutamine, nonessential amino acids, penicillin, streptomycin, and 5% fetal bovine serum. In some cases, before incubation, T-cells were removed by exposure to rabbit anti-mouse theta sera in the presence of rabbit complement. Dilutions of *E. corrodens* LPSs, OMFs, and controls of concanavalin A and *Salmonella typhi* LPS were added at the time the cells were cultured. Cells were pulse-labeled with 1 μCi of [^{3}H]thymidine for 48 h and harvested 18 to 24 h later. The amount of radioactivity incorporated into acid-precipitable material was determined by scintillation counting. Each test was run in triplicate, and the mitogenic activity was expressed as a stimulation index: the amount of radioactivity incorporated into test (treated) cells (mean counts of triplicate wells) divided by the amount of radioactivity incorporated into untreated wells. A stimulation index equal to or greater than 4 is considered significant (i.e., mitogenic) and a stimulation index less than 2 is considered insignificant (i.e., not mitogenic). Stimulation indices between 2 and 4 were not interpretable.

RESULTS

Mitogenic activity of *E. corrodens* cellular fractions. The mitogenic activities of the *E. corrodens* OMFs and LPSs were determined in spleen cells of both BDF and C3H/HeJ (LPS-nonresponder) mice. Both OMFs were potent mitogens for BDF spleen cells (Table 1); doses between 5 and 5×10^{-5} μg/ml induced a significant mitogenic response in BDF splenocytes. The *E. corrodens* LPS was also mitogenic for BDF spleen cells at a concentration as low as 0.05 μg/ml. Concanavalin A and *S. typhi* LPS, used as positive controls, were both potent mitogens for the BDF cells.

TABLE 1. Mitogenic effects of *E. corrodens* OMF and LPS on whole spleen cells of BDF mice

Dose (μg/ml)	Stimulation index[a]				
	Strain 23834		Strain 470 OMF	ConA[b]	LPS[c]
	LPS	OMF			
50	5.6	18.5	59.4	70.8	50.5
5	15.3	32.2	44.1		
0.5	16.2	13.1	15.1		
0.05	5.8	5.7	5.4		
0.005	3.9	4.4	6.0		
0.0005	3.3	4.0	6.2		
0.00005	2.8	4.0	4.4		

[a] Stimulation index (counts per minute for treated culture/counts per minute for culture with medium alone); 4 is significant activity.
[b] Concanavalin A; 2 μg/ml.
[c] *S. typhi* LPS; 1 μg/ml.

When doses of the *E. corrodens* OMFs were incubated with spleen cells of C3H/HeJ mice (LPS-nonresponder strain), the mitogenic activities were reduced (Table 2), as they should be in this mouse strain, but were still significantly higher than that of the noninducing LPS. In fact, even high doses (5 and 50 μg/ml) of the *E. corrodens* LPS failed to mitogenically stimulate C3H/HeJ cells. The C3H/HeJ cells also did not respond to the classical *S. typhi* control LPS, but did respond mitogenically to concanavalin A.

Since LPS is exclusively a B-cell mitogen (3), and since the *E. corrodens* LPS was not mitogenic for C3H/HeJ murine spleen cells (Table 2), the *E. corrodens* 23834 OMF was tested in cultures of C3H/HeJ spleen cells that had been depleted of T-lymphocytes to determine whether the OMF was mitogenic for B-cells by a mechanism independent of LPS (Table 3). When T-lymphocyte-depleted C3H/HeJ spleen cells were cultured with dilutions of strain 23834 OMF, a dose-response for mitogenic stimulation resulted; the minimally effective concentration was 5 μg/ml. This is comparable to that for whole spleen cells, (0.5 μg/ml, minimum dose) and indicates that the *E. corrodens* strain 23834 OMF is a B-cell mitogen by a mechanism different from that elicited by conventional LPS.

Bone resorptive activity of *E. corrodens* cellular fractions. Since loss of tooth-supporting alveolar bone is a cardinal feature of periodontitis (17), the effect of the *E. corrodens* OMFs and LPSs on bone resorption was determined. Various membrane fractions were examined (Table 4). The low-speed pellet after French pressure cell disruption strongly stimulated resorption, giving a response somewhat greater than that of *E. coli* LPS. Membrane fractions derived during subsequent purification steps contained less activity; however, the membrane fractions both before and after Triton X-100 treatment still stimulated significant bone resorption, similar to that for *E. coli* LPS.

Stimulation by *E. corrodens* LPS and OMFs were compared to that produced by *S. typhi* LPS and *E. coli* LPS (Table 5), two classical LPSs that are known to stimulate resorption. Although the OMFs from both strains stimulated ^{45}Ca release, the activity associated with strain 470 was minimal. The LPS from strain 23834 caused a nearly twofold augmentation of ^{45}Ca release from treated bone. This response was equivalent to that elicited by the *S. typhi* LPS or the *E. coli* LPS.

TABLE 2. Mitogenic effects of *E. corrodens* OMF and LPS on whole spleen cells of C3H/HeJ mice[a]

Dose (μg/ml)	Stimulation index				
	Strain 23834		Strain 470 OMF	ConA	LPS
	LPS	OMF			
50	1.3	8.9	26.9	90.3	2.8
5	1.1	11.7	15.8		
0.5		4.0	5.8		
0.05	0	2.6	1.9		

[a] Headings and abbreviations as in Table 1.

***Limulus* amebocyte lysate clotting activity of *E. corrodens* cellular fractions.** The clotting or gelation of *Limulus* amebocyte lysate is reportedly induced by LPS and can be used as a measure of endotoxic activity (1). When the *E. corrodens* LPSs and OMFs were assayed by this method, as little as 0.062 μg of *E. corrodens* LPS induced amebocyte lysate clotting, as compared to 0.031 μg required for the control *E. coli* LPS (Table 6). Interestingly, the OMFs of both strains 23834 and 470 induced gelation at even lower concentrations, 0.015 μg, and are thus more active than the *E. corrodens* LPSs in inducing amebocyte lysate clotting.

DISCUSSION

The relatively high activity of the *E. corrodens* 23834 OMF and purified LPS in an in vitro bone resorption model suggests that both of these components have the potential to stimulate bone resorption in vivo. In fact, the LPS from *Salmonella* species as well as from periodontal disease-associated organisms have previously been shown to be active stimulators of bone resorption (7; B. C. Nair and E. Hausmann, J. Dent. Res. Spec. Issue A, vol. 60, abstr. 132 F, 1981). Nair and Hausmann, for example, have reported that very low concentrations of LPS from a strain of *Bacteroides gingivalis* stimulated resorption even in submicrogram amounts. In addition, Kiley and Holt (12) have observed that the purified LPS from *A. actinomycetemcomitans* is also a potent stimulator of bone resorption. Further evidence that LPS may be one of the important mediators of resorption is provided by an animal model system with ligature-induced periodontitis in *Macaca arctoides* monkeys (6). A monkey immunized with a homogeneous preparation of *B. gingivalis* LPS exhibited greatly reduced bone loss as compared with an unimmunized control animal. Thus, it appears that antibody to LPS inhibited the ligature-induced bone resorption.

Since there is likely a reduced content of LPS in the OMF of strain 23834 due to Triton X-100 extraction (15), the bone-resorptive activity of the OMF is due to either the remaining LPS or even to one or more other outer membrane components. A synergistic effect of the LPS and other outer membrane components in enhancing the stimulation of bone resorption is possible. One possible candidate for this effect is outer membrane-associated peptidoglycan especially since a portion of it still remains associated with the OMFs (15). Synthetic muramyl dipeptide, the repeating unit of the peptidoglycan disaccharide backbone, has been shown to be a potent stimulator of bone resorption (B. Alander, K. Nuki, and L. S. Raisz, Calcif. Tissue Int. vol. 31, abstr. no. 1, 1980). In addition, even low concentrations of LPS have been demonstrated (16) to act synergistically with other bone-resorbing agents to produce a much enhanced bone resorptive response as compared with the sum of the two acting independently. This ability of biological molecules to act synergistically could have important implications in the periodontal pocket, since there is a strong association between the peptidoglycan and outer membrane in gram-negative bacteria, and the two components are thus likely to be exposed to the periodontal tissues as a complex of macromolecules. This may be of considerable importance since the bacteria in the periodontal pocket are probably in a continuous process of growth, death, and lysis and shedding pieces of cell wall (including outer membrane and peptidoglycan) into the gingival sulcus. Whether other outer membrane components such as outer membrane porins have synergistic effects similar to those of LPS is not known at this time.

It appears that the *E. corrodens* LPS from strain 23834 is as active as "classical" enteric LPS in this bone-resorptive assay system, and that the strain 23834 OMF is only slightly less so, whereas strain 470 OMF is relatively inactive. The absence of bone-resorptive activity of the strain 470 OMF is perplexing, but may indicate structural or compositional differences between this outer membrane and that of strain

TABLE 3. Mitogenic effects of *E. corrodens* 23834 OMFs on T-lymphocyte-depleted spleen cells of C3H/HeJ mice[a]

Dose (μg/ml)	Stimulation index		
	Strain 23834 OMF	ConA	LPS
50	33.3	2.4	1.1
5	11.4		
0.5	3.7		
0.05	1.3		
0.005	1.5		

[a] Headings and abbreviations as in Table 1.

TABLE 4. In vitro bone resorptive activities of *E. corrodens* 23834 cell fractions

Fraction	^{45}Ca release (treated/control ratio)[a]
Low-speed pellet[b]	2.33 ± 0.20[c]
Pre-Triton X-100 membrane fraction	1.94 ± 0.11[c]
OMF	1.77 ± 0.15[c]
Triton X-100-soluble fraction	1.58 ± 0.24
E. coli LPS	1.72 ± 0.27[c]

[a] Values are means ± standard errors for six pairs of bones cultured for 48 h.
[b] Each test preparation was at a final concentration of 3 μg/ml.
[c] Significantly different from 1.0 ($P < 0.05$).

TABLE 5. In vitro bone-resorptive activities of *E. corrodens* LPSs and OMFs

Prepn[a]	^{45}Ca release (treated/control ratio)[b]
E. corrodens 470 OMF	1.20 ± 0.07[c]
E. corrodens 23834 OMF	1.77 ± 0.05[c]
E. corrodens 23834 LPS	1.95 ± 0.23[c]
S. typhi LPS	1.98 ± 0.17[c]
E. coli LPS	2.17 ± 0.31[c]

[a] Each test preparation was at a final concentration of 3 μg/ml.
[b] Values are means ± standard errors for six pairs of bones cultured for 48 h.
[c] Significantly different from 1.0 ($P < 0.05$).

TABLE 6. *Limulus* amebocyte lysate clotting activity of *E. corrodens* cellular fractions

Test sample size (μg)	*Limulus* lysate clotting activity[a]			
	E. coli LPS	Strain 23834		Strain 470 OMF
		LPS	OMF	
0.125	+	+	+	+
0.062	+	+	+	+
0.031	+	±	+	+
0.015	−	−	+	+
0.0075	−	−	±	±

[a] +, Solid clot in bottom of tube; ±, gelation that slid down sides of tube; −, no gelation.

23834. These differences may be due either to a difference in in vivo cellular structure of the two strains or to subtle differences in the purification of the two preparations. We have previously noted both chemical and morphological differences between the OMF of both *E. corrodens* strains (15).

The data reported here demonstrate that both the LPSs and OMFs of *E. corrodens* are potent mitogens for murine lymphocytes, although the LPS is less potent than the OMFs. The higher potency of the OMFs for spleen cells of BDF mice as compared with that of the LPS, seems to indicate that another component of the OMFs besides LPS, possibly an LPS-associated protein, is mitogenic. This is more clearly indicated by the mitogenicity studies with spleen cells from C3H/HeJ mice, a strain that does not respond mitogenically to LPS due to a single autosomal gene defect (26). Even a high dose (50 μg/ml) of the *E. corrodens* LPS (or 1 μg of *S. typhi* LPS per ml) failed to stimulate the C3H/HeJ cells. However, submicrogram amounts of either OMF produced significant stimulation. Thus, some component of the OMFs, other than LPS, must be mitogenic for C3H/HeJ spleen cells. One or more of the *E. corrodens* outer membrane proteins may very well be mitogenic, since Chen and co-workers (5) have reported that three outer membrane proteins of *Pseudomonas aeruginosa* are mitogenic for C3H/HeJ spleen cells, when tested under conditions similar to those described here. One of the mitogenic outer membrane proteins of *P. aeruginosa* is the porin protein F. Thus, considering the large amount of the *E. corrodens* porin-like protein (15) in the OMFs, the porin may be a major mitogenic component of the OMFs.

The fact that the *E. corrodens* OMF is also mitogenic for T-lymphocyte-depleted spleen cells of C3H/HeJ mice suggests that the OMF is a B-cell mitogen by a mechanism other than LPS or conventional lipid A mitogenicity. These results are also similar to those reported by Chen et al. (5) for the *P. aeruginosa* outer membrane porin.

It thus appears that in spite of containing a nonclassical endotoxic structure, i.e., little 2-keto-3-deoxyoctulosonic acid and heptose and no detectable β-hydroxy fatty acids (15), the *E. corrodens* LPS does, in fact, exhibit classical endotoxic activities. Such activities include bone resorption, mitogenic stimulation of BDF, but not C3H/HeJ, spleen cells, and *Limulus* amebocyte lysate clotting. These results confirm and further expand two previous studies of biological activities of *E. corrodens* LPS (2; S. Sasaki and I. Takazoe, J. Dent. Res. IADR Spec. Issue D, abstr. no. 8, vol. 58, 1979). Sasaki and Takazoe isolated the LPSs from *E. corrodens* strains 1073 and 1080 and found both to be as active as *E. coli* O111:B4 LPS in the production of skin lesions, pyrogenicity, and *Limulus* lysate clotting. Behling and co-workers (2) isolated LPS from two variants of *E. corrodens* strain 1073 and also found the LPSs to be very active in *Limulus* lysate clotting, Shwartzman skin reactivity, and chicken embryo lethality bioassays. Unfortunately controls of reference LPS and the biochemistry of these isolated LPS preparations were not reported and therefore preclude any structural comparisons between these *E. corrodens* LPS preparations and the *E. corrodens* 23834 LPS.

The connective tissue destruction characteristic of periodontitis may result either directly or indirectly (via the host response) from the effects of bacterial products or bacterial cell components (or both) that readily penetrate into the gingival tissue. Schwartz et al. (19), for example, have reported that radiolabeled LPS can pass into and through intact gingival epithelium in dogs. It is conceivable that LPS with associated outer membrane components, such as peptidoglycan and outer membrane proteins or even entire pieces or blebs of outer membrane, could penetrate an intact gingiva. The studies reported here provide evidence that the *E. corrodens* bacterial surface structures, once in the gingival tissue, would be "primed" to activate tissue-destructive mechanisms, either directly or by stimulation of the host's immune response.

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