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Biological Activity of the Slime and Endotoxin of the Periodontopathic Organism *Eikenella corrodens*

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A partially purified water-soluble slime extract was obtained from two strains of the oral pathogen *Eikenella corrodens*, designated CS10 A and CS10 B. Endotoxin was also isolated from this organism by the phenol-water extraction procedure. Assays including the local Shwartzman skin reactivity, chicken embryo lethality, *Limulus* lysate clotting, spleen cell mitogenicity, and immune adjuvancy were used to test the biological properties of these two bacterial extracts. In each of these assay systems the endotoxins of the *Eikenella corrodens* strains demonstrated the classical endotoxic responses. In contrast, the effect of slime extract in the corresponding assays was either extremely low or absent with the exception of a very strong immunosuppressive effect. Mice treated with slime extract showed a severely reduced immune response to sheep erythrocytes as measured by the hemolytic plaque-forming cell assay.

It is well known that bacteria or their microbial products can modulate the immune mechanism as exogenous immunoregulatory agents. Presentation of bacterial cells to a host, either as infection or vaccine, exposes the host, in addition to the immunogens, to a variety of pharmacologically active components which may induce immunopotentiation and immunosuppression. Not surprising, therefore, is that immunosuppressive and potentiating activities can often be demonstrated in the same bacterial preparation (1, 2, 6). As we and others have demonstrated, even a purified bacterial cell wall extract, such as endotoxin, and its nontoxic polysaccharide moiety can exhibit both immune adjuvancy or suppression, depending upon the time of administration (4, 7, 12).

Eikenella corrodens, a gram-negative anaerobic organism, was isolated from a human periodontitis lesion by Newman et al. (15). The strain (isolate no. 1073) that was kindly provided for these studies by S. Socransky has been found to give rise to two morphological variants. One, designated as CS10 A, grows on blood agar under anaerobic conditions as small, shiny, raised colonies. The other, designated CS10 B, shows under identical conditions a dull, spreading, agar-corroding growth. Ultrastructure and biochemical characterization of *E. corrodens* CS10 have been reported by C.-H. Lai, M. A. Listgarten, A. Crawford, and S. S. Socransky (J. Dent. Res., Abstr. no. 1004, 1976) and by A. C. R. Crawford, S. S. Socransky, C. Stone, M. A. Listgarten, and C.-H. Lai (J. Dent. Res., Abstr. no. 1005, 1976). Electron microscopic sections of the bacteria revealed only minor differences between the two strains. The cell wall structure of CS10 B is more convoluted than that of CS10 A. Also, CS10 B cells are longer than those of CS10 A, and their cytoplasm is slightly more electron dense. Antigenically, these two strains are nearly identical. In our own investigations, animals immunized with phenol-killed whole bacteria of either strain CS10 A or CS10 B show nearly identical serum titers against both strains. Further evidence that these two strains are highly related and may share a common origin comes from the observation that strain CS10 B under prolonged cultivation on blood agar spontaneously and unidirectionally converts to CS10 A.

It was found by Socransky and associates that *E. corrodens* mono-infected rats develop severe alveolar bone resorption. Further details were subsequently reported by A. C. R. Crawford, S. S. Socransky, E. Smith, and R. Phillips (J. Dent. Res., Abstr. no. 275, 1977). These observations were confirmed by us in a subsequent study in which germ-free rats infected with CS10 A and CS10 B showed evidence of severe bone lesions (10). The histopathological changes of the rat gingiva during the development of the disease have been described by us in a recent publication (14). In brief, periodontal-like disease in the gnotobiotic rat is characterized by a progressive destruction of the interdental tissues, including gingiva and alveolar bone, which may occur in the absence of an inflammatory response. With progress of the disease, crater-like defects de-

velop in the alveolar bone around and between the molars. Yet despite these effects, no increase in the number of inflammatory cells in the connective tissue is detectable. Even in later stages of the development of periodontal lesions the gingival connective tissue remains relatively free of typical lymphocytes and plasma cells.

We isolated several cell wall components and cell products from *E. corrodens* and studied their possible roles in the pathogenesis of the experimental periodontal lesions. We report here the findings obtained by using the endotoxin and the slime preparations in various immune biological parameters.

MATERIALS AND METHODS

Animals. (i) Mice: 12- to 16-week-old female ICR mice (ICR, Philadelphia, Pa.) were used where indicated. (ii) Rabbits: 10- to 12-week-old New Zealand white rabbits (Rockland Farms, Gilbertsville, Pa.) were used for the Shwartzman skin reaction. (iii) Chicken embryo: 11-day-old chicken embryos (Shaw Hatcheries, West Chester, Pa.) were used for the toxicity assay.

Cultivation of *E. corrodens*. Bacteria were grown in 5-liter volumes in which the following components were dissolved in distilled water: 85 g of Trypticase; 15 g of Phytone; 20 g of NaCl; 5 g of potassium nitrate; and 5 g of sodium formate. After autoclaving for 20 min, 15 mg of hemin dissolved in 100 ml of distilled water was added to the culture medium. Each flask was inoculated with 50 ml of a 24-h culture grown in the above-described medium. The bacteria were grown at 37°C for 48 h without agitation. At this time samples were taken from each flask and cultured on blood agar plates to assure a pure culture. The bacteria grown in batch cultures were killed with 0.5% phenol, collected by centrifugation at 1,000 × *g* for 30 min, and washed once with distilled water.

Preparation of bacterial sonic extracts. A suspension of killed (0.5% phenol, final concentration) bacteria containing 10^8 to 10^9 cells in distilled water was sonically disrupted with a Branson Sonifier on ice for 15 min at 100 W.

Isolation of endotoxic lipopolysaccharides. Harvested, washed, and lyophilized bacteria (1 g) were extracted with 50 ml of 45% phenol at 70°C by the method of Westphal and Lüderitz (18). The water phase was dialyzed at 4°C for 3 days against distilled water, filtered, and precipitated by 2 volumes of methanol containing 0.1% $MgCl_2$. The precipitate was redissolved in 10 ml of distilled water and sedimented by ultracentrifugation at 100,000 × *g* for 2 h. The sediment was lyophilized. The same procedure was applied to both CS10 A and B strains.

Preparation of slime extract. After repeated washing and centrifugation, a 10-g wet bacterial cell pellet was resuspended in 2 volumes of 0.15 M NaCl. The cell suspension was subsequently placed in a Waring blender and homogenized for 3 min at very low speed. After centrifugation at 6,000 × *g* for 30 min, the supernatant containing the crude slime was removed. Extraction of slime from this supernatant was done by the method of Bartell et al. (3). In brief, the supernatant containing the crude slime extract was dialyzed with three changes of distilled water for 48 h before being treated with 3 volumes of ethanol in the presence of sodium acetate (10%) and glacial acetic acid (1%) at 4°C. The resulting precipitate was collected by centrifugation, suspended in distilled water, and again dialyzed. Trichloroacetic acid was added to the dialyzed material to a final concentration of 10%, and the precipitate that formed was removed by centrifugation. The supernatant containing the slime was dialyzed for 48 h and lyophilized. The yields were 121 mg and 96 mg for CS10 A and CS10 B, respectively.

Biological assays. (i) *Limulus* assay. The *Limulus* lysate clotting assay was carried out by the procedure of Levin et al. (13). Accordingly, 50 μl of each test dilution was placed in 5-ml pyrogen-free borosilicate glass culture tubes. To these an equal volume of 50 μl of reconstituted *Limulus* lysate (Associates of Cape Cod, Woods Hole, Mass.) was added. After brief mixing, the tubes were placed in a 37°C water bath and left undisturbed for 30 min. Gelation (as determined by inverting the tube twice) was considered a positive endpoint. Pyrogen-free saline served as a negative control.

(ii) Shwartzman assay. A slightly modified technique of the original Shwartzman procedure was used (16). Accordingly, abdomens of 5- to 7-pound (ca. 2.3- to 3.2-kg) New Zealand white rabbits were shaved and zoned by means of a dye pen into equal areas. Test preparations were injected intradermally in fixed concentrations in 0.1 ml of saline with a 26 gauge needle. After 24 h the endotoxin (20 μg) challenge was given intravenously in 0.2 ml of saline. The surface area of the nearly circular necrotic lesions was calculated from the average of two perpendicular diameters.

(iii) Chicken embryo lethality. After careful removal of a small section of the outer eggshell, 11-day-old chicken embryos were injected intravenously with doses of 0.1, 1.0, and 10 μg of the preparation in 0.1 ml of pyrogen-free saline. After a 24-h incubation period, the eggs were cracked open to determine survival. The 50% lethal dose was determined according to the Spearman-Karber method (16).

(iv) Lymphoblast response of sensitized mice. Mice (six per group) were immunized intraperitoneally three times at weekly intervals with 200-μg doses of whole bacterial cell sonic extracts in 0.2 ml of incomplete Freund adjuvant. One week after the third and last immunization, spleen cells were obtained by teasing the organs in a small volume of RPMI 1640 medium enriched with 10% fetal calf serum (GIBCO, Grand Island, N.Y.). Penicillin-streptomycin (GIBCO; 100 U) was added. The cells were filtered through sterile gauze and adjusted to a concentration of 2 × 10^6 viable cells per ml. One milliliter of this suspension was added to triplicate tubes that contained one of the following preparations: 10 μg of slime from CS10 A or CS10 B and 10 μg of endotoxin from CS10 A or CS10 B; 10 μg of concanavalin A (Sigma Chemical Co., St. Louis, Mo.); or saline. The culture tubes were maintained at 37°C for 68 h in a 5% CO_2 incubator, after which they received 1 μCi of tritiated thymidine (New

England Nuclear Corp., Boston, Mass.) in 10 ml of saline. The tubes were then incubated for an additional 4 h and harvested as described previously (10). Radioactivity incorporation into the cells was expressed as the counts per minute of the sample divided by the counts per minute of the saline controls, giving a stimulation index.

(v) Plaque-forming cell assay. A modified technique of the hemolytic plaque assay first described by Jerne and Nordin (9) was used. Again, mice in groups of six were used. Spleens were obtained 4 days after immunization with 10^8 sheep erythrocytes, and spleen cell suspensions were adjusted to three concentrations (by volume, 1:10, 1:100, and 1:500) for plating. For an accurate determination of cell concentration, samples of these cell suspensions were stained and counted by means of a hemacytometer. Two-milliliter aliquots of a 1% Noble agar solution containing 0.1 ml of 1% diethylaminoethyl-dextran, 0.1 ml of a 20% sheep erythrocyte suspension, and 0.1 ml of any of the various spleen cell concentrates were evenly layered on plates containing 10 ml of 1.4% solidified but prewarmed Noble agar. The plates were incubated for 60 min at 37°C before 2.5 ml of guinea pig complement, diluted 1:10 with Veronal buffer, was added. The plates were incubated for an additional 30 min. At this time the complement was decanted and the plates were ready to by analyzed. Plates for each of the three concentrations of spleen cells were done in triplicate and analyzed in a blind manner.

RESULTS

Table 1 summarizes the results of the *Limulus* lysate clotting assay, Shwartzman skin reactivity, and chicken embryo lethality. Although the endotoxin from the CS10 A strain showed the same activity as other gram-negative endotoxins would, the toxicity of the CS10 B strain was lower than usual. In contrast, the slimes of *E. corrodens* failed to elicit any of these endotoxic reactions.

Of interest was whether these bacterial cell wall products contributed to the induction of cell-mediated immunity. One of the most quantitative methods of assessing cell-mediated immunity involves the in vitro exposure of spleen cells from previously immunized animals to the same antigen(s) in vitro. The incorporation of [^{3}H]thymidine after several days in culture reflects the degree of antigenically induced lymphoblast transformation of thymus-derived lymphocytes. The results in Table 2 indicate the degree of lymphoblast transformation in response to the slimes and endotoxins of *E. corrodens*. As the data clearly indicate, there was no significant stimulation for any of the preparations when the spleens of nonimmunized animals were used, but when the spleen cells of previously immunized animals were tested, good stimulation was observed for the endotoxins of both strains. The slime extracts, however, still failed to exert any stimulatory effects.

Changes in the humoral immune response to sheep erythrocytes induced by these preparations were tested by the hemolytic plaque assay (Table 3). The major point of interest is the contrasting effects of the endotoxin and slime of both strains. Whereas the endotoxins of CS10 A and CS10 B provided good immune potentiation, their respective slimes proved to be severely immunosuppressive.

TABLE 1. *Assay results*

Material	*Limulus* lysate clotting (μg)[a]	Shwartzman skin reactivity (cm^2)[b]	Chicken embryo lethality (LD_{50} in μg)[c]
E. corrodens CS10 A			
Slime	≫0.1	0.049	>10
Endotoxin	0.0001	1.72	0.135
E. corrodens CS10 B			
Slime	≫0.1	0	>10
Endotoxin	0.001	0.67	0.150

[a] Lowest concentration of the preparation which still resulted in positive clotting.

[b] Size of skin hemorrhage elicited by 10 μg of material given intradermally.

[c] Fifty percent lethal dose (LD_{50}) value represents that dose which, when injected intravenously, produces 50% lethality.

DISCUSSION

In previous studies we attempted to correlate the development of the immune response to *E. corrodens* with the severity and rate of appearance of macroscopic periodontal lesions in gnotobiotic rats (10, 11). A possible inverse relation-

TABLE 2. *In vitro lymphoblast transformation induced by endotoxin and slime of E. corrodens in normal and sensitized spleen cells*

Prepn[a]	Lymphoblast transformation			
	Normal spleen cells		Sensitized spleen cells	
	Activity (cpm)[b]	SI[c]	Activity (cpm)	SI
CS10 A				
Slime	1,071	0.97	3,210 ± 234	0.99
Endotoxin	1,833 ± 129	1.06	14,413 ± 318	4.43
CS10 B				
Slime	1,730 ± 139	1.01	3,909 ± 209	1.20
Endotoxin	2,054 ± 113	1.20	19,640 ± 584	6.04
Saline control	1,716 ± 142		3,909 ± 162	
Concanavalin A	34,188 ± 687	19.09	33,252 ± 543	10.22

[a] Ten micrograms of each preparation was added to each culture tube.

[b] Activity in counts per minute (cpm) represents the average value of six spleens tested in triplicate ± one standard error.

[c] Stimulation index (SI) is the activity of the treated group over the activity of the saline control group.

TABLE 3. *Humoral immune suppression and enhancement of humoral response to sheep erythrocytes[a] by the slime and endotoxin of E. corrodens*

Prepn[b]	PFC per spleen[c] (×10⁴)	SI[d]
CS10 A		
Slime	0.70 ± 0.17	0.23
Endotoxin	14.50 ± 1.51	4.74
CS10 B		
Slime	0.05 ± 0.02	0.016
Endotoxin	11.45 ± 0.93	3.74
Saline control	3.06 ± 0.32	

[a] Sheep erythrocytes were injected intraperitoneally, 10^8 per mouse.

[b] Twenty micrograms of each preparation was injected intraperitoneally.

[c] Plaque-forming cell (PFC) response per spleen is the average of six spleens tested in triplicate ± one standard error.

[d] Stimulation index (SI) is the number of plaque-forming cells of the treated group over the plaque-forming cell response of the saline control.

ship was found in which a strong cell-mediated immune response as measured by skin reactivity and lymphocyte mitogenesis occurred in the first few weeks after infection and subsided soon thereafter. Furthermore, humoral antibodies to endotoxin from *E. corrodens* could not be detected at any time of the disease process. The disease developed only after the cell-mediated immune response subsided, thus suggesting that a lack of an efficient immune response may permit the development of the disease.

The major point of interest that can be drawn from the data presented here is that different bacterial cell wall components of the same microorganism can have contrasting effects on the immune-regulatory mechanisms. Whereas endotoxin proved to be a good adjuvant and strongly induced lymphoblast transformation, the slime extract, particularly from the CS10 B strain of *E. corrodens*, proved to be very immunosuppressive in the anti-sheep erythrocyte plaque-forming cell response. In addition, the slime extract was unable to induce lymphoblast transformation. This immunosuppressive effect of the slime extract of *E. corrodens* seems to parallel the observations that the extracellular slime polysaccharide of *Pseudomonas aeruginosa* exerts immunosuppressive properties, which were shown in several studies by Bartell and co-workers to reduce (i) humoral immune response, (ii) cell-mediated immunity, and (iii) phagocytosis; in addition, the slime induced leukopenia in which polymorphonulcear leukocytes were totally absent (3, 5, 17). This comprehensive immunosuppressive effect of the extra-cellular slime provides a tentative explanation to the histopathological and immunological anomaly in which *E. corrodens*-induced bone destruction in gnotobiotic rats proceeds in the absence of (i) significant levels of inflammation and (ii) detectable serum antibody levels against the endotoxin of *E. corrodens*.

Chemical analysis of the slime extracts from *E. corrodens* is presently in progress in our laboratory.

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