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Bacteriological Study of Periodontal Lesions in Two Sisters with Juvenile Periodontitis and Their Mother

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A total of five bacteriological samples from the periodontal pockets of two sisters with localized juvenile periodontitis and their mother with advanced periodontitis was studied. Gram-negative anaerobic rods were predominant in the samples. *Bacteroides intermedius* and *Bacteroides loescheii* were the most predominant species. The antigenicity and bacteriocinogenicity of these isolates were quite similar. Serum immunoglobulin G antibody levels of the subjects to gram-negative periodontopathic bacteria were measured by using the micro-enzyme-linked immunosorbent assay. The levels of antibodies to saccharolytic black-pigmented *Bacteroides* species were significantly higher than the levels in healthy young females.

Localized juvenile periodontitis (LJP) is characterized by deep periodontal pockets and bone loss localized around the first molar and incisors without subgingival calculus (1). Microbial observations of the periodontal pockets of juvenile periodontitis have revealed that the lesions consist predominantly of gram-negative bacteria, including *Actinobacillus actinomycetemcomitans* and *Capnocytophaga* species (8, 16, 17, 19). It has also been demonstrated that patients with LJP have depressed neutrophil chemotaxis (2, 3, 7, 20).

In the present study we describe the predominant cultivable flora of samples from the periodontal pockets of two sisters with LJP and their mother. The serum immunoglobulin G (IgG) antibody levels to oral gram-negative bacteria were measured by using the micro-enzyme-linked immunosorbent assay.

MATERIALS AND METHODS

Subjects. Two sisters aged 14 and 19 years with LJP and their mother with advanced periodontitis were the subjects of the present study. These patients had not received any antibiotics within the previous 4 months. Eight young females (age range, 18 to 23 years) in a healthy periodontal condition (no evidence of gingival inflammation or periodontal pockets) were used as controls for serum samples.

Sampling and media. The sampling sites were lesions with at least 5 mm of loss of attachment by means of a probe and 4 mm of bone loss. Before sampling, supragingival plaque was removed with sterile scalers and cotton rolls. Subgingival plaque samples were taken with sterilized paper points (standardized 30; Maillefer, Switzerland). The paper point was inserted into the apical subgingival region and left for 10 s and then removed under CO_2 gas-flush conditions.

The samples were immediately transferred into 10 ml of Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) containing glass beads. The medium was shaken vigorously for 1 min to disperse the sample. A portion of dispersed sample was observed microscopically according to the method of Listgarten and Helleden (9). Serial 10-fold dilutions were made in an anaerobic chamber containing 10% CO_2, 10% H_2, and 80% N_2. From each dilution, 0.1-ml samples were plated on nonselective and selective media.

Trypticase soy agar (BBL) supplemented with 10% horse blood, hemin (5.0 μg/ml), and menadione (0.5 μg/ml) was used for nonselected anaerobic and aerobic growth. Selective media for black-pigmented *Bacteroides* species (10), *Eikenella corrodens* (C. Walker, A. C. R. Tanner, C. Smith, and S. S. Socransky, J. Dent. Res., Special issue 57A, abstr. no. 961, p. 315, 1978), *A. actinomycetemcomitans* (12), *Fusobacterium nucleatum* (21), and *Capnocytophaga* species (13) and MS medium (Difco Laboratories, Detroit, Mich.) for streptococci were used to isolate the various species. Selective media for *A. actinomycetemcomitans* and *Capnocytophaga* species were cultured under 20% CO_2–80% air. After 7 to 9 days of incubation, dilutions containing about 50 colonies were chosen for further study.

To obtain immunogens and sonicated antigens, a diffusate broth was used. Thirty-four grams of Trypticase peptone (BBL) and 6 g each of Phytone peptone (BBL) and yeast extract (Difco) were dissolved in 150 ml of distilled water. This preparation was dialyzed against 850 ml of distilled water for 2 days at 4°C. After the volume of diffusate was adjusted to 1 liter, 5 g of NaCl, 2.5 g of K_2HPO_4, and 2.5 g of glucose were added, the pH was adjusted to 7.4, and the medium was autoclaved. Afterward, a sterilized solution of 5 mg of hemin–0.5 mg of menadione was added aseptically.

Identification. Colonies on each plate were picked, purified, and characterized. Colony morphology, gram-stain morphology, motility, and aerobic growth were checked. Indole production, esculin hydrolysis, nitrate reduction, gelatinase activity, catalase, and fermentation of glucose, lactose, sucrose, cellobiose, and mannitol were tested. Acid end products were determined by gas liquid chromatography. The isolate identification was mainly based on data from Holdeman et al. (5).

Immunodiffusion. Rabbit antisera were prepared against whole cells of *Bacteroides gingivalis* 381 (originally isolated from a periodontal pocket by S. S. Socransky, Forsyth Dental Center, Boston, Mass.), *Bacteroides intermedius* 20-3 (isolated by J. Slots, State University of New York at Buffalo, N.Y.) and *Bacteroides loescheii* ATCC 15930. Cells grown in the diffusate broth were washed three times with phosphate-buffered saline (PBS) (pH 7.2) and suspended at

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10 mg (wet weight) per ml. Antisera were obtained after repeated intravenous injections of the immunogen.

One percent agarose (Wako Chemicals, Tokyo, Japan) dissolved in 0.03 M Veronal buffer (pH 8.6) was used for single immunodiffusion experiments. Cells grown in the diffusate broth were sonicated at a 100-W output with a flat microtip in suspensions placed in ice-cold water. Sonicate supernatants were obtained by centrifugation at 12,000 × *g* for 20 min. Sonicated soluble antigen of the tested strain was placed in the wells and antiserum was placed in the troughs and allowed to diffuse at room temperature overnight.

Bacteriocin activity. Bacteriocin activity of black-pigmented *Bacteroides* isolates from the family samples was examined by the stab culture method by using Trypticase soy agar plates supplemented with hemin (5.0 μg/ml) and menadione (0.5 μg/ml).

Measurement of serum IgG antibodies to gram-negative periodontopathic bacteria. Micro-enzyme-linked immunosorbent assay (4) was used to measure specific IgG titer against sonicates from *B. gingivalis* 381, *B. intermedius* 24 and JPY 14, *B. loescheii* ATCC 15930, *F. nucleatum* ATCC 25586, *E. corrodens* FDC 1073, *A. actinomycetemcomitans* ATCC 29522 and Y4, and *Capnocytophaga* sp. strains M-12 and 25. These antigens were prepared by ultrasonication of cells from 48- to 72-h cultures of each strain. The sonicate supernatants were dialyzed against water.

A 200-μl sample of ultrasonic antigens derived from the sonicate supernatant suspended at 10 μg/ml in 0.1 M carbonate buffer (pH 9.6) was placed in each well of a flat-bottom microplate (Dynatech Laboratories, Inc., Alexandria, Va.). After incubation at 37°C for 2 h, the plates were thoroughly washed with PBS (pH 7.2) containing Tween 20. A 200-μl sample of a twofold dilution of serum sample in PBS was added to each well. After incubation at 37°C for 2 h, the plate was washed with PBS. A 200-μl sample of a 1,000-fold dilution of goat anti-human IgG (heavy-chain specific; Sigma Chemical Co., St. Louis, Mo.) which was conjugated to alkaline phosphatase (Sigma) was added to each well and incubated at 37°C for 1 h. After the wells had been washed three times with PBS, a 200-μl sample of *p*-nitrophenylphosphate (Sigma) suspended in a 0.05 M sodium carbonate buffer (pH 9.8) containing 1 mM $MgCl_2$ was added to each well and incubated at room temperature for 30 min. After the reaction was stopped by the addition of 50 μl of 1 N NaOH, the absorbance at 410 nm was determined in a micro-enzyme-linked immunosorbent assay reader (MR 590; Dynatech). All measurements were performed in duplicate.

The dilution and the absorbance were plotted on the X and Y axes, respectively, and the straight line of the regression was drawn. The antibody titer was expressed as $\log_2$ at the intersection of 0.8 absorbance and the regression line.

RESULTS

The proportions of cultivable bacteria and spirochetes in subgingival samples are summarized in Table 1. From each sample, 30 to 67 colonies were picked and subcultured on Trypticase soy blood agar. A total of 265 isolates were examined from the five samples. Of the isolates, 30% (79 strains) grew in aerobic cultures. Gram-negative organisms ranged from 55 to 84% of cultivable flora. Saccharolytic black-pigmented colonies were isolated from all sites examined and averaged 38% of the total cultivable colonies. *B. gingivalis* was not isolated from any sample tested. Isolates categorized as unidentified *Bacteroides* species in Table 1 were anaerobic gram-negative rods which were difficult to identify or were lost on subculture before identification.

TABLE 1. Microbial flora in periodontal pockets

Bacterial species	% Of cultivable microbiota				
	Younger sister		Elder sister		Mother
	[6 site	2] site	[6 site	1] site	[5 site
Bacteroides intermedius	27.8	44.2	8.9	10.8	13.3
Bacteroides loescheii	14.8	8.3	27.5	14.5	20.0
Bacteroides oralis				3.6	
Unidentified *Bacteroides* sp.			7.8	7.3	
Selenomonas sputigena			1.8		
Unidentified motile rod			1.8		
Fusobacterium nucleatum		30.4	7.8	5.5	13.3
Fusobacterium mortiferum					3.3
Capnocytophaga sp.	31.5	0.3	3.9	9.1	6.7
Actinobacillus actinomycetemcomitans	0.05	0.3			
Eikenella corrodens	3.7		10.7	3.6	
Veillonella sp.	3.7		5.9		
Neisseria sp.				1.8	
Actinomyces viscosus	5.6	2.8	5.9	14.5	6.7
Actinomyces naeslundii	1.9		2.0	9.1	6.7
Rothia dentocariosa			3.9		
Nocardia species	1.9				
Arachnia propionica					6.7
Propionibacterium acnes				3.6	
Eubacterium sp.			2.0		10.0
Clostridium perfringens					3.3
Unidentified aerobic gram-positive rod					3.3
Streptococcus sanguis	3.7	8.3	5.9	3.6	
Streptococcus mitis	1.9	2.8	3.9	7.3	3.3
Streptococcus salivarius	3.7	2.8		1.8	
Peptococcus saccharolyticus					3.3
Unidentified gram-positive cocci				3.6	
Spirochetes (microscopic)	23.4	29.6	27.1	17.6	48.8

Other gram-negative rods, including *E. corrodens*, *F. nucleatum*, and *Capnocytophaga* species, were also isolated. Gram-positive rods constituted 16 to 45% of the total isolates and were mostly *Actinomyces* species.

Microscopically, spirochetes were predominant in all samples, especially in the sample from the mother with advanced periodontitis.

An immunodiffusion pattern between the antigens of the *B. intermedius* and *B. loescheii* isolates and antiserum to black-pigmented *Bacteroides* species is shown in Fig. 1. Antigens of each *B. intermedius* isolate from the family formed several precipitin lines to antiserum against this species. Antigens of *B. loescheii* reacted to antiserum for the species. These antigens did not react to the antisera for different species.

All saccharolytic black-pigmented *Bacteroides* isolates had identical inhibitory spectra against black-pigmented *Bacteroides* species isolated from the human oral cavity. All *B. intermedius* and *B. loescheii* isolates from the family showed strong inhibitory activity against five strains of *B. gingivalis* studied. These isolates exhibited weak inhibition to one strain of *B. intermedius* and no inhibition to four other strains of the species isolated from human subgingival plaque samples in our laboratory. The isolates did not show any inhibition to five strains of *B. loescheii*. It was confirmed that all black-pigmented *Bacteroides* isolates did not inhibit the growth of those species isolated from the lesions of the family.

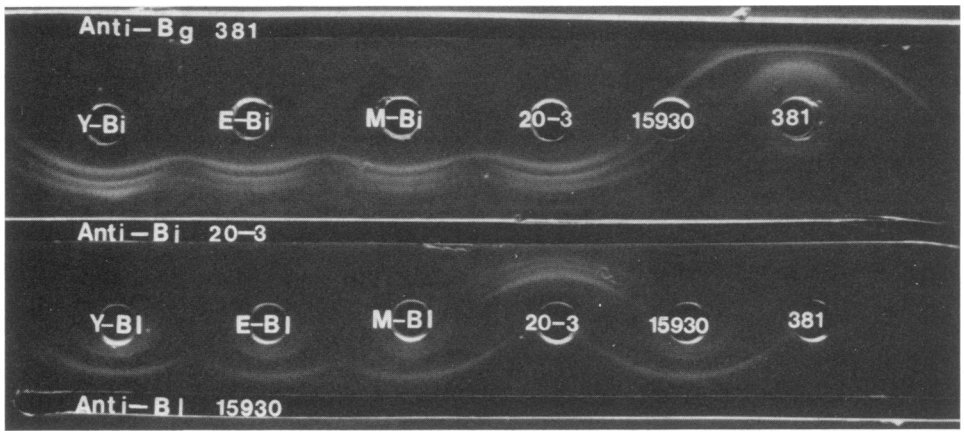

FIG. 1. Immunodiffusion of sonicated antigens of *B. intermedius* and *B. loescheii* that were isolated from the family reacted with antisera to black-pigmented *Bacteroides* species. The wells contain the sonicate supernatant of cells from *B. intermedius* isolated from the younger sister (Y-Bi), the elder sister (E-Bi) and their mother (M-Bi), *B. loescheii* isolated from the younger sister (Y-BI), the elder sister (E-BI), and their mother (M-BI), *B. loescheii* (ATCC 15930), *B. intermedius* (20-3), and *B. gingivalis* (381). Troughs contain antiserum to *B. gingivalis* 381 (Anti-Bg), *B. intermedius* 20-3 (Anti-Bi), and *B. loescheii* (Anti-BI), respectively.

The specific IgG antibody levels to 10 strains of 7 species in the sera from the family and mean titers of eight females (aged 18 to 23 years) are shown in Fig. 2. The specific IgG levels to *B. intermedius* strains were higher than the levels to antigens of other species, but no significant differences were found between the sera from the family and those from the healthy group. The levels of antibody to *B. loescheii* in sera from the family were higher than the mean level of healthy young females. There were no marked differences in the antibody levels to *F. nucleatum*, *E. corrodens*, *A. actinomycetemcomitans*, and *Capnocytophaga* species between the family and healthy females.

DISCUSSION

The present study demonstrated that the predominant organisms in these familial lesions of LJP and advanced periodontitis were *B. intermedius* and *B. loescheii*. The findings suggest that these saccharolytic black-pigmented *Bacteroides* species are contributing to the disease process in the family. Previous data have shown that these species increase in lesions of pregnancy gingivitis (6), experimental gingivitis (18), acute necrotizing ulcerative gingivitis (11), and young adult periodontitis (14). These saccharolytic black-pigmented *Bacteroides* species are some of the main pathogens of these periodontal diseases.

The antibody responses of the family to *B. intermedius* seem to correlate well with the levels of this species in the periodontal pocket. This implies that there is no humoral immunological defect. We could not detect any abnormality of polymorphonuclear leukocyte chemotaxis, immunological defects, or other systemic disorders in these patients.

A. actinomycetemcomitans was detected in samples from the younger sister on the selective medium, but the proportions of the species in the microbial flora were only 0.05 to 0.3%. The sonicated antigen of *A. actinomycetemcomitans* isolates reacted to antiserum against *A. actinomycetemcomitans* ATCC 29522. No colonies of *A. actinomycetemcomitans* were detected in the samples from the elder sister or the mother. Serum IgG antibody levels to *A. actinomycetemcomitans* in the family were lower than in healthy young females. In addition, the sera from the family did not react to sonicated antigens of *A. actinomycetemcomitans* ATCC 29522, ATCC 29523, ATCC 29524, and Y4 in 1% agarose. These findings indicate that *A. actinomycetemcomitans* was not prominently associated with periodontal pathology in these patients.

B. intermedius and *B. loescheii* strains isolated from the family all belonged to the same serogroups, and they exhibited identical inhibitory spectra to black-pigmented *Bacteroides* species. These results suggest maternal and intrafamilial transfer of these organisms. The melaninocin activity designated by Nakamura et al. (15) might be one of the factors which inhibit *B. gingivalis* colonization in periodontal pockets. To confirm this possibility, further studies of bacteriocin-like activity of periodontopathic bacteria are required.

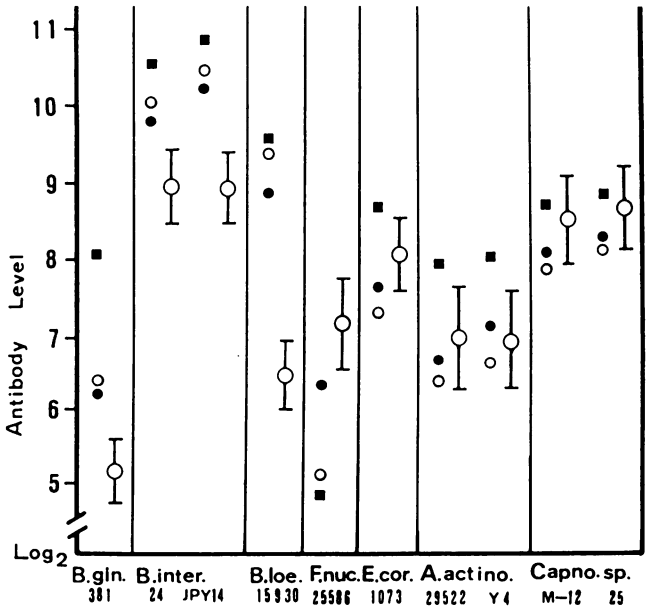

FIG. 2. Levels of serum IgG antibodies to 10 strains of gram-negative bacteria. Symbols: ○, younger sister; ●, elder sister; ■, their mother; ⊻, mean titer and standard error for eight healthy females aged 18 to 23 years.

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