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Longitudinal Study of Experimentally Induced Periodontal Disease in *Macaca arctoides*: Relationship Between Microflora and Alveolar Bone Loss

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Macaca arctoides monkeys develop periodontal disease, and they harbor a periodontopathic indigenous flora largely similar to that of humans. This study showed that various *Haemophilus* isolates and H_2O_2-splitting asaccharolytic *Bacteroides melaninogenicus* strains constituted major segments of the monkey periodontal microflora. These organisms have not been previously identified among human isolates. Furthermore, the present data revealed that asaccharolytic *B. melaninogenicus* strains increased in proportion from a few percent to about 66% of the total isolates concomitant with the development of a significant loss of alveolar bone mass. Hence, this study strongly implicates *B. melaninogenicus* subsp. *asaccharolyticus* and closely related strains as important pathogens in actively destructive periodontal disease.

The infectious nature of human periodontal disease is well established, and recent studies have shown distinct differences in the microflora of the healthy and diseased periodontium (16, 25, 26). Briefly, the healthy gingival sulcus harbors a scant microflora dominated by gram-positive organisms (85%), usually *Actinomyces* and *Streptococcus* species. In the apical part of deep periodontal pockets in adults, *Bacteroides melaninogenicus* subsp. *asaccharolyticus* (proposed classification, *Bacteroides asaccharolyticus* [3]), *Fusobacterium nucleatum*, and other gram-negative anaerobic rods make up about 75% of the cultivable microflora. The deep-pocket microflora in patients with juvenile periodontitis is also made up mainly of gram-negative organisms (about 65%), but is of a nature different from that of adult periodontitis, being predominated by isolates of *Bacteroides* species, such as *Bacteroides ochraceus* (*Capnocytophaga*) and other organisms of unknown species.

The available microbiological data on periodontal disease, however, all originate from cross-sectional examinations. Therefore, these studies cannot establish to what extent the different organisms initiate or are secondary to the pathological changes. One approach to further define periodontal pathogens involves longitudinal examination of the periodontal microflora throughout disease development. However, a longitudinal study of the microflora associated with human periodontal disease involves several difficulties. Problems exist in predicting if and when a gingivitis lesion converts into a progressive periodontitis lesion with periodontal fiber destruction and loss of alveolar bone. Furthermore, the periodontal disease process may include periods of exacerbation and remission with possible concomitant changes in the periodontal microflora. These reasons, together with the lack of a standard clinical method for determining the rate of progression of the periodontal disease at a given moment, have impeded the designations of organisms as "contaminating" or periodontopathic in periodontal disease. Also, ethical considerations can restrict the use of humans in longitudinal periodontitis studies.

Development of a periodontal disease model in *Macaca arctoides* (*Macaca speciosa*) would be of particular value for investigation of the periodontal microflora during disease development for the following reasons. (i) *M. arctoides* monkeys seem to develop periodontal disease under natural conditions somewhat similar to those in humans (13). (ii) The placement of a silk ligature around the crown of a tooth can induce loss of alveolar bone within a period of 2 to 3 weeks. This alveolar bone loss is due to bacterial action and not to a mechanical trauma of the ligature; thus, antibacterial therapy before and during the presence of the ligature will prevent alveolar bone loss (E. Hausmann, L. Ortman, and N. Sedransk, manuscript in preparation). (iii) Many of the periodontal organisms of *M. arctoides* may be closely related to human isolates (29).

The aims of the present study were to examine the subgingival bacterial flora of *M. arctoides*

during an experimental periodontitis study and to determine how the subgingival microbial composition related to the clinical status of the periodontal tissues.

MATERIALS AND METHODS

Animals. Two female *M. arctoides* monkeys (no. 7397, 8 kg in weight; no. 7444, 7 kg in weight) were used in this study. Before this experiment, both monkeys were healthy and housed individually in our animal facilities in a room with other monkeys. They were fed a primate diet in hard biscuit form. The monkeys had their permanent dentitions fully erupted. During the measurement and sampling procedures used in this experiment, the animals were anesthetized with a mixture of phencyclidine hydrochloride and promazine.

Experimental design. A summary of the experimental design is outlined in Table 1. After the first bacteriological sample, the consistency of the diet was changed by adding enough water to the biscuits to make them soft. The soft-diet regime was maintained throughout the remaining part of the study. On day 7, a 3-0 silk ligature was placed around the crown of the left lateral maxillary incisor. The ligature was positioned supragingivally on the distal, lingual, and buccal surfaces and subgingivally mesially and secured to the tooth in such a way that it did not mechanically damage the periodontal fiber attachment. After the first 28 to 35 days of the study, the monkeys were treated subsequentially for 2 to 3 weeks with 250 mg of tetracycline a day given in the drinking water and by daily rinsing of the ligated area with a solution of 0.2% chlorhexidine gluconate.

No mechanical oral hygiene procedures, such as tooth brushing and interdental flossing, were carried out in any experimental phases.

Clinical measurements. Routine clinical measurements of the mesial periodontal pocket of the left lateral maxillary incisor, including Silness and Löe plaque index (22), Löe and Silness gingival index (17), and periodontal pocket depth, were carried out throughout the study. In addition, the ^{125}I absorptiometric technique developed by C. O. Henrikson was used to quantitate changes in the alveolar bone mass (7, 8). This technique measures the bone mass at the same alveolar bone site with a precision of 1 to 3%. The same position of the intraoral ^{125}I source relative to the alveolar bone site and to an extraoral scintillation detector was maintained by means of a precision-made occlusal splint. The bone measurements were made immediately below the crest of the alveolar bone septum between the left central and the lateral maxillary incisor.

Sample collection. Bacteriological specimens were collected by using six sterile fine absorbent paper points (Johnson & Johnson) which were inserted sequentially into the periodontal pocket, each being retained therein for 10 s. The paper points were transferred to a test tube containing 1.5 ml of prereduced, anaerobically sterilized (PRAS) Ringer solution. Before the sampling, the gingival margin and the supragingival tooth surface were carefully cleansed of saliva, debris, and plaque with sterile gauze. The sampling took place under anaerobic conditions by flushing the

TABLE 1. *Experimental design and clinical data of a longitudinal study of experimentally induced periodontitis in two M. arctoides monkeys*[a]

Clinical data	Monkey	Pre-	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
Bacteriological sampling	7397		+	+			+				+
	7444										
Dietary regime	7397	Hard	Soft								
	7444										
Ligature	7397										
	7444										
Tetracycline and chlorhexidine	7397										
	7444										
Plaque index[b]	7397		2	2			2				2
	7444		2	3			3				3
Gingival index[b]	7397		1	2			2				2
	7444		1	2			3				3
Bone mass in mg/mm^2 and the % change in bone mass denoted in parentheses[b]	7397		1.353 (0)	1.323 (−2)	1.338 (−1)	1.357 (0)	1.208 (−11)	1.074 (−21)	1.138 (−16)	1.143 (−16)	1.390 (3)
	7444		1.022 (0)	0.916 (−10)	0.892 (−13)	0.654 (−36)	0.630 (−38)	0.910 (−11)	1.018 (0)	1.101 (8)	0.734 (−28)

[a] The periodontal pocket depth was 0.5 to 1.5 mm for both monkeys throughout the study.
[b] The figures denote the results obtained for the test periodontal pockets.

sampling area with a mixture of 85% Ar, 10% H_2, and 5% CO_2.

Bacteriological processing. The sample material was mixed by a Vortex mixer for 60 s. Serial 10-fold dilutions were then made in tubes of PRAS Ringer solution. Portions (0.1 ml) of appropriate dilutions of the specimens were plated on Trypticase soy agar (Baltimore Biological Laboratory, Cockeysville, Md.) supplemented with 10% sheep blood, 5 μg of hemin per ml, and 0.5 μg of menadione per ml. Plating and primary incubation took place in an anaerobic chamber (Coy Manufacturing Co., Ann Arbor, Mich.). The blood agar plates were stored for at least 12 h in the anaerobic glove box before inoculation. In addition, a phase-contrast microscopic examination of the original sample material was performed in a Petroff-Hausser counting chamber to determine the total cell counts per milliliter and the percentages of motile rods and spirochetes in the sample.

Isolation and identification. Colonies were picked at random from blood agar plates containing well-separated colonies after 7 days of incubation. Culture purity of the isolates was checked by repeated subcultivations. Each pure culture was identified by Gram stain characteristics, morphology, anaerobiosis, and key biochemical reactions according to established procedures (1, 9, 30). A total of 21 colonies were lost on primary subculture, and, therefore, these strains were not identified.

The following biochemical tests were performed routinely for each category of isolates: (i) gram-positive facultative cocci—hydrogen peroxide decomposition, esculin and arginine hydrolyses, growth at 10°C and in the presence of 6.5% NaCl, extracellular polysaccharide formation from sucrose, and acid production from basal medium, esculin, raffinose, glucose, inulin, mannitol, and sorbitol; (ii) gram-positive anaerobic cocci—volatile and nonvolatile acid production in PYG medium (9), hydrogen peroxide decomposition, esculin and gelatin hydrolyses, indole production, nitrate reduction, and acid production from basal medium, cellobiose, esculin, fructose, glucose, lactose, sucrose, and maltose; (iii) gram-positive facultative and anaerobic rods—volatile and nonvolatile acid production in PYG medium, motility, hydrogen peroxide decomposition, esculin and gelatin hydrolyses, indole production, nitrate reduction, and acid production from basal medium, cellobiose, esculin, glucose, lactose, mannitol, raffinose, sorbitol, trehalose, and xylose; (iv) gram-negative anaerobic cocci—volatile and nonvolatile acid production in PYG medium, hydrogen peroxide decomposition, nitrate reduction, and acid production from basal medium, glucose, and maltose; (v) gram-negative facultative rods—volatile and nonvolatile acid production in PYG medium, X-factor and V-factor requirements, motility, hydrogen peroxide decomposition, oxidase test, esculin and urea hydrolyses, indole and hydrogen sulfide production, nitrate and nitrite reduction, oxidative/fermentative metabolism of glucose, and acid production from basal medium, esculin, glucose, lactose, mannitol, sucrose, and xylose; (vi) gram-negative anaerobic rods—volatile and nonvolatile acid production in PYG medium, motility, hydrogen peroxide decomposition, esculin hydrolysis, indole production, nitrate reduction, growth in the presence of 2% oxgall, and acid production from basal medium, arabinose, cellobiose, esculin, fructose, glucose, maltose, mannitol, mannose, melezitose, raffinose, rhamnose, trehalose, and xylose. The basal medium utilized was that described by S. S. Socransky, A. C. R. Crawford, S. Sasaki, M. J. Lescord, and C. R. Proule (J. Dent. Res., Special Issue B, AADR Abstr. no. 279, 1977) for some isolates supplemented with 1% hemolyzed blood as a growth stimulant. On the basis of the routine biochemical tests listed, the isolates were divided into groups and classified as to genera and for some strains also as to species. For bacterial groups of uncertain taxonomic position, additional biochemical tests were carried out on representative strains to obtain a more comprehensive characterization.

RESULTS

Six monkeys were studied for their alveolar bone response to the placement of a ligature in the periodontal pocket area. A significant bone loss was observed for all monkeys between 2 and 3 weeks after the ligature application with the ^{125}I absorptiometric technique (Hausmann et al., manuscript in preparation). This paper includes microbiological analysis for two representative monkeys of this group.

A series of clinical examinations of the mesial aspects of the ligated left maxillary lateral incisor of these two monkeys was carried out during the 8-week experimental period (Table 1).

The amounts of supragingival dental plaque in close proximity to the gingival margin of the test periodontal pocket showed no marked differences for both monkeys at the four registration times listed in Table 1. However, the severity of the gingival inflammation increased distinctly, and a significant loss of alveolar bone mass was found after the introduction of the soft-diet regime and the insertion of the silk ligature (Table 1). This was found for both monkeys, although the rate of progression of the periodontal destruction and the severity of the periodontal disease obtained were greater in monkey no. 7444 than in monkey no. 7397. The combined clinical data suggested that the soft diet-silk ligature combination aggravated the periodontal disease by allowing the establishment of a periodontal microflora of a higher pathogenicity.

Four bacteriological samples from the test periodontal pocket in each monkey were examined over an 8-week period (Table 1). A total of 24 to 52 isolates were characterized from each periodontal pocket sample. On the basis of Gram stain, morphology, and anaerobiosis, the monkey subgingival bacteria were grouped into the bacterial categories shown in Table 2. The classification of the organisms recovered as to genus and species is presented in Table 3.

TABLE 2. *Initial grouping of the subgingival microorganisms in a longitudinal study of experimentally induced periodontitis in two M. arctoides monkeys*

Category[a]	% of total counts							
	Monkey no. 7397				Monkey no. 7444			
	Week 0	Week 1	Week 4	Week 8	Week 0	Week 1	Week 4	Week 8
Gram-positive facultative cocci[b]	8	6	2	ND[c]	75	7	ND	ND
Gram-positive anaerobic cocci[b]	19	6	ND	4	ND	7	2	ND
Gram-positive facultative rods[b]	8	8	2	13	ND	15	13	4
Gram-positive anaerobic rods[b]	36	17	10	ND	ND	11	ND	ND
Gram-negative anaerobic cocci[b]	ND	4	ND	ND	ND	ND	ND	ND
Gram-negative facultative rods[b]	11	42	ND	29	23	24	2	42
Gram-negative anaerobic rods[b]	18	18	85	54	2	37	82	58
Vibrio-Selenomonas-like organisms[d]	19	57	18	20	23	18	30	26
Spirochetes[d]	8	5	13	10	10	10	7	0
Total cell counts $\times 10^4$/ml[d]	61	210	440	255	84	77	1,215	163
Total no. of isolates characterized[b]	47	52	41	24	48	46	45	24

[a] No isolates of gram-negative facultative cocci.
[b] Data obtained from culture studies.
[c] ND, Not detected among the isolates comprising >2 to 4% of the cultivable microflora.
[d] Data obtained from phase-contrast microscopic examination of the original sample material.

At week 0, the subgingival microflora of both monkeys was dominated by gram-positive organisms which constituted approximately three-fourths of the total isolates (Table 2). *Eubacterium* species and *Peptostreptococcus/Peptococcus* species predominated in monkey no. 7397, and *Streptococcus* species were the predominating isolates in monkey no. 7444 (Table 3).

The subgingival microbial composition of both monkeys changed markedly between week 0 and week 1 after the change to a soft-diet regime. This microbial change was accompanied by the development of a more severe gingival inflammation and loss of alveolar bone mass (Table 1). In the 1-week sample, gram-negative species constituted almost two-thirds of the total monkey subgingival isolates (Table 2). The proportional increase of the gram-negative isolates in monkey no. 7397 was mainly due to an increase in *Haemophilus*-like organisms (Table 3). The greatest loss of alveolar bone mass was found in monkey no. 7444 (Table 1). The subgingival microflora of this monkey showed a marked increase in the proportions of *B. melaninogenicus* subsp. *asaccharolyticus*, *Bacteroides capillosus*, and *Vibrio succinogenes* (Table 3).

The presence of silk ligature altered the subgingival microflora. At week 4, gram-negative organisms comprised more than 80% of the total isolates, and the great majority of the isolates were members of *B. melaninogenicus* (Table 3). In monkey no. 7397, *B. melaninogenicus* subsp. *asaccharolyticus* strains constituted 51% of the total isolates, and H_2O_2-splitting, nonfermentative or weakly fermentative *B. melaninogenicus* isolates constituted 15% of the total isolates. Monkey no. 7444 exhibited the greatest loss of alveolar bone mass also at week 4 (Table 1). In this monkey, *B. melaninogenicus* subsp. *asaccharolyticus* strains comprised 5% of the total isolates, and the H_2O_2-splitting *B. melaninogenicus* strains made up 63% of the cultivable microflora.

The antibacterial therapy affected to some extent the subgingival microflora of monkey no. 7397. Gram-negative species constituted more than 80% of the subgingival microflora also in the 8-week sample (Table 2), but, interestingly, the asaccharolytic strains of *B. melaninogenicus* comprised only 8% of the total isolates (Table 3). The clinical data for monkey no. 7397 at week 8 revealed that the bone loss had stopped and that a remineralization of the alveolar bone was taking place (Table 1). In monkey no. 7444, at week 8 the subgingival microflora sampled closely resembled that of week 4 with respect to a strong predominance of H_2O_2-splitting *B. melaninogenicus* strains (Table 3); however, spirochetes were not detected in the 8-week sample from monkey no. 7444 (Table 2). Bone breakdown reappeared in this monkey after a previous period of bone regeneration during the time of antibacterial therapy (Table 1).

The present study gave rise to the recovery of organisms which were difficult to identify as to species according to current classification schemes. A significant segment of the microflora in almost every sample was gram-negative facultative rods. These isolates generally had a dry, rough, irregular-edged, adherent colony on

TABLE 3. *Predominant subgingival microflora in a longitudinal study of experimentally induced periodontitis in two M. arctoides monkeys*

Monkey	Week 0	Week 1	Week 4	Week 8
7397	6% *Streptococcus sanguis*	4% *S. sanguis*	2% *S. sanguis*	
	2% *Streptococcus milleri*	2% *S. milleri*		
	19% *Peptostreptococcus/ Peptococcus* sp.	6% *Peptostreptococcus/ Peptococcus* sp.		4% *Peptostreptococcus/ Peptococcus* sp.
	4% *Actinomyces naeslundii*			13% *A. naeslundii*
	4% *Actinomyces viscosus*			
		2% *Arachnia propionica*	5% *A. propionica*	
		6% Gram-positive facultative rod		
	36% Asaccharolytic *Eubacterium* sp.	17% Asaccharolytic *Eubacterium* sp.	7% Asaccharolytic *Eubacterium* sp.	
		4% *Veillonella parvulae*		
	11% *Haemophilus* sp.	45% *Haemophilus* sp.		21% *Haemophilus* sp.
	2% *Vibrio succinogenes*	2% *V. succinogenes*		
		2% *Selenomonas sputigena*		
		4% *Fusobacterium nucleatum*	15% *F. nucleatum*	29% *F. nucleatum*
	4% Corroding *Bacteroides* sp.			8% Corroding *Bacteroides* sp.
			2% *Bacteroides bivius*	
		8% *Bacteroides* sp.		4% *Bacteroides* sp.
			2% *B. melaninogenicus* subsp. *intermedius*	13% *B. melaninogenicus* subsp. *intermedius*
			51% *B. melaninogenicus* subsp. *asaccharolyticus*	
	11% H_2O_2-splitting *Bacteroides melaninogenicus*	2% H_2O_2-splitting *B. melaninogenicus*	15% H_2O_2-splitting *B. melaninogenicus*	8% H_2O_2-splitting *B. melaninogenicus*
7444	52% *S. sanguis*	2% *S. sanguis*		
	19% *S. milleri*			
	2% *Streptococcus mitior*			
	2% *Staphylococcus/Micrococcus* sp.	7% *Staphylococcus/Micrococcus* sp.		
		7% *Peptostreptococcus productus*		
		13% Gram-positive facultative rod	15% Gram-positive facultative rod	4% Gram-positive facultative rod
		11% *Asaccharolytic Eubacterium* sp.		
			3% *Propionibacterium acnes*	

7444	23% *Haemophilus* sp.	24% *Haemophilus* sp.	3% *Haemophilus* sp.	36% *Haemophilus* sp.	
		13% *V. succinogenes*			
			3% *S. sputigena*		
	2% *F. nucleatum*	4% *F. nucleatum*	3% *F. nucleatum*	4% *F. nucleatum*	
		9% *Bacteroides capillosus*			
			8% *Bacteroides* sp.		
		11% *B. melaninogenicus* subsp. *asaccharolyticus*	5% *B. melaninogenicus* subsp. *asaccharolyticus*	4% *B. melaninogenicus* subsp. *asaccharolyticus*	
			63% H_2O_2-splitting *B. melaninogenicus*	52% H_2O_2-splitting *B. melaninogenicus*	

blood agar plates, and their growth in broth medium was granular with a strongly adherent sediment to the bottom of the test tube. The Gram stain showed short, thin coccobacilli, often in pairs. On the basis of the biochemical reactions listed in Table 4, 12 representative strains were divided into two groups. The 12 strains were included in the *Haemophilus* genus, even though requirements of hemin and nicotinamide adenine dinucleotide were not obvious on subculture. They did not fit any recognized species, but seemed closely related to the *H. aphrophilus* group (12).

This study permitted the isolation of *B. melaninogenicus* strains which instantly decomposed 3% hydrogen peroxide when a slide test was performed on colonies carefully removed from the Trypticase soy blood agar plates. This hydrogen peroxide decomposition, suggesting catalase activity, is a feature which previously has not been attributed to the *B. melaninogenicus* species. The colonies of the H_2O_2-splitting *B. melaninogenicus* isolates were grayish-green colored in early culture, turning black with prolonged incubation, and contained coccobacillary cells which exhibited some pleomorphism in broth culture. The H_2O_2-splitting *B. melaninogenicus* isolates exhibited many biochemical

TABLE 4. *Properties of 12 gram-negative facultative rods[a] isolated from M. arctoides subgingival microflora*

Test	Results in:	
	7 Strains	5 Strains
Hemolysis	−	−
Motility	−	−
H_2-O_2 splitting	+	+
X-factor requirement	−	−
V-factor requirement	−	−
Oxidase	−	−
Esculin hydrolysis	−	−
Urease	−	−
Indole production	+	−
H_2S production from cystein	+	+
Nitrate reduction	+	+
Nitrite reduction	−	−
Ornithine decarboxylase	−	−
Lysine decarboxylase	−	−
Oxidative/fermentative metabolism	F	F
pH, basal medium	7.1	6.8
pH, 0.25% esculin	7.1	6.8
pH, 1% glucose	5.5	5.3
pH, 1% lactose	5.8	6.8
pH, 1% mannitol	7.3	6.9
pH, 1% sucrose	7.1	6.8
pH, 1% xylose	6.3	5.9
Metabolic acid end products	A, P, iv, b, ib, l, s	a, l, s

[a] Originating from a total of six periodontal pockets in two *M. arctoides*. Abbreviations: F, fermentative; a, acetic acid; P, propionic acid; iv, isovaleric acid; ib, isobutyric acid; b, butyric acid; l, lactic acid; s, succinic acid.

TABLE 5. *Properties of 10 H_2O_2-splitting strains of B. melaninogenicus[a] isolated from M. arctoides subgingival microflora*

Test	Results
Hemolysis	−
H_2O_2 splitting	+
Hydrogenase	−
Starch hydrolysis	−
Esculin hydrolysis	−
Arginine hydrolysis	−
Gelatin liquefaction	+
Urease	−
Indole production	+
H_2S production from cystein	+
Nitrate reduction	−
2% Oxgall (bile) growth	−
Pyruvate utilization	+
pH, basal media	6.9
pH, 1% glucose	6.1
pH, 1% mannose	6.2
No acid production from:	Amygdalidin, adonitol, cellobiose, dextrin, dulcitol, esculin, fructose, inositol, maltose, mannitol, melezitose, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, starch, trehalose, xylose
Metabolic acid end products	A, P, ib, b, iv, s

[a] Originating from a total of seven periodontal pockets in two *M. arctoides*. See footnote *a* of Table 4 for abbreviations.

characteristics of *B. melaninogenicus* subsp. *asaccharolyticus* (Table 5). However, the H_2O_2-splitting *B. melaninogenicus* strains lowered the pH value by 0.7 to 0.8 unit in glucose and mannose broths. A similar pH change was not found for the non-H_2O_2-splitting asaccharolytic *B. melaninogenicus* strains isolated in the present study. Also, the H_2O_2-splitting *B. melaninogenicus* strains produced more propionic acid (>1 meq/100 ml) and less butyric acid (<1 meq/100 ml) in glucose broth cultures than usually found for human oral *B. melaninogenicus* subsp. *asaccharolyticus* strains. Furthermore, none or very little fluorescence of the H_2O_2-splitting *B. melaninogenicus* cells was observed by an indirect immunofluorescent technique using rabbit antiserum to a human oral strain of *B. melaninogenicus* subsp. *asaccharolyticus*. This antiserum regularly gives a strongly positive fluorescent reaction with homologous human oral isolates of *B. melaninogenicus* subsp. *asaccharolyticus* (S. Sasaki, J. Slots, B. Hammond, and S. S. Socransky, J. Dent. Res., Special Issue A, IADR Abstr. no. 966, 1978).

DISCUSSION

Animal models have been widely used in the study of periodontal disease (19). Rodents with a suppressed flora and germfree rodents have been infected with organisms isolated from the human oral cavity. Experimental rodents can develop periodontal disease within a few weeks; however, the rodents and humans differ markedly in morphology of the tooth-periodontal complex, the oral microflora, and the immunological response, factors which can make it difficult to relate the rodent results to human periodontal disease.

Beagle dogs, marmoset monkeys, squirrel monkeys, *M. arctoides*, chimpanzees, and baboons have served as models in chronic periodontitis studies. The periodontal disease process of higher animals supposedly mimics relatively closely that of human periodontal disease. Unfortunately, these experimental animals, including *M. arctoides* (13), often require months or even years to develop a significant loss of alveolar bone. Application of a ligature around the crown of a tooth adjacent to the alveolar bone, however, can induce alveolar bone loss in beagle dogs and monkeys within a period of a few weeks (6, 11, 15). The inflammatory infiltrate of the periodontal tissue of the ligated tooth seems dominated by polymorphonuclear leukocytes (6), whereas the "common" human periodontal disease is characterized by the predominance of lymphocytes and plasma cells (20). The ligature-related periodontal disease, accordingly, appears both in time and histopathologically more acute than the typical human periodontal disease. However, human periodontal disease may proceed by periods of rapid bone loss and by periods of remission. The microbiological results of the present study, therefore, may relate to human periodontal disease in its exacerbated phases.

The use of *M. arctoides* in the study of periodontal disease seems justified by the present microbiological data. The monkey periodontal pockets harbor several species which have been isolated from the human periodontium. The present study could not establish with the few monkeys examined the extent to which the mutual proportions of the subgingival bacterial species may differ between monkeys and humans. Also, different biotypes may inhabit monkeys and humans. The recovery of the *Haemophilus* and the H_2O_2-splitting *B. melaninogenicus* monkey isolates may exemplify this.

The subgingival microflora of the two monkeys studied changed profoundly during the 8-week experimental period, but the pattern of the microbial changes was in many ways similar for the two monkeys. The introduction of the soft-diet regime increased the gram-negative species population with a magnitude of about twofold and also initiated a more severe gingival inflammation. These data confirm previous clinical findings in *M. arctoides* on the gingivitis-stimulating effect of a soft-diet regime (28), and they agree well with human studies which point to

gram-negative organisms as potential pathogens in gingivitis (25). Data exist on the effect of a dietary regime on the gram-positive dental plaque flora (31), but little is known about the importance of dietary factors for the oral colonization of gram-negative organisms. It may be hypothesized that a soft-diet regime enhances the accumulation of gram-positive dental plaque bacteria which may serve as attachment sites for various gram-negative organisms (24).

The ligature-related alveolar bone loss was associated with a subgingival microflora strongly predominated by the asaccharolytic strains of *B. melaninogenicus*. At week 4, these isolates comprised 66 and 68% of the total isolates from monkey no. 7397 and monkey no. 7444, respectively (Table 3). *B. melaninogenicus* subsp. *asaccharolyticus* strains were the most numerous isolates in monkey no. 7397 (51%), whereas the H_2O_2-splitting *B. melaninogenicus* strains were predominant in monkey no. 7444 (63%). These data indicate a periodontopathic potential of both types of *B. melaninogenicus*. They also parallel the finding in a recent study of advanced human adult periodontitis in which *B. melaninogenicus* subsp. *asaccharolyticus* isolates averaged about one-third of the cultivable subgingival microflora (23). Further studies are necessary to determine the reason for the proportional increase of *B. melaninogenicus* concomitant with the insertion of the ligature. The possibility exists that the ligature, by mechanical irritation of the gingival tissues, induces an exudation into the periodontal pocket area of tissue fluid which may stimulate the growth of *B. melaninogenicus*. This possible irritation of the gingival tissue will not, by itself, lead to a recognizable alveolar bone breakdown within the experimental period utilized in the present study. Thus, unpublished data from our laboratory showed that an antibiotic treatment during the ligated period will inhibit loss of alveolar bone. It is also conceivable that the ligature allows *B. melaninogenicus* cells to proliferate in the periodontal pocket somehow protected from the cleansing forces of the gingival fluid flow and thereby increase their numbers in the periodontal pocket.

The antibacterial therapy initiated in both monkeys a distinct bone regeneration during the time of therapy (Table 1). In monkey no. 7397, in which the bone remineralization also continued throughout the first postantibacterial week, the asaccharolytic *B. melaninogenicus* isolates made up only 8% of the cultivable subgingival microflora at week 8 (Table 3). This proportion of *B. melaninogenicus* strains was lower than that of the base line sample at week 0. The effect of the antibacterial therapy in monkey no. 7444 seemed limited to the period of the administration of the antibacterial agents. Already during the first week after the termination of the antibacterial treatment, approximately 33% of the alveolar bone mass was lost at the localized site beneath the alveolar crest studied. The 8-week subgingival microflora associated with this bone loss was comprised mainly of the H_2O_2-splitting strains of *B. melaninogenicus* (Table 3).

A significant finding in the present study was the demonstration of a strong positive relationship between the number of *B. melaninogenicus* isolates and the amount of alveolar bone loss. This study, therefore, greatly incriminates *B. melaninogenicus* subsp. *asaccharolyticus* as an important pathogen in periodontal disease. That *B. melaninogenicus* is a potential pathogenic organism is also suggested from studies of nonoral infections. *B. melaninogenicus* has been isolated from postoperative wound infections of the abdomen, abdominal wall infections, peritonitis, and many other infections of the intestinal tract and is one of the most common species encountered in pleuropulmonary infections and in serious infections of the female genital tract (2).

It seems clear that strains of *B. melaninogenicus*, including strains of identified subspecies, are found in association with many infections, including infections in the oral cavity. However, the mechanism(s) whereby these microorganisms actually contribute to the pathology associated with them is not well understood.

An indication of the infectious role played by *B. melaninogenicus* comes from studies in which bacterial samples taken from the oral cavity were inoculated subcutaneously into guinea pigs, resulting in the formation of necrotic lesions. The infectious flora was a mixture of organisms, but *B. melaninogenicus* was always an indispensable component of each infectious mixture (18, 27). Furthermore, several strains of *B. melaninogenicus* elaborate various cytotoxic products and proteolytic enzymes, including a collagenase, which are potentially damaging to the periodontal tissues (4). Also, the endotoxin of *B. melaninogenicus* has been shown by Hausmann et al. (5) to stimulate bone resorption in vitro. A direct pathogenic role of *B. melaninogenicus* is also indicated in the study of Ingham et al. (10). They found that phagocytosis and killing of *Proteus mirabilis* by human polymorphonuclear leukocytes was inhibited in vitro by *B. melaninogenicus* and other obligately anaerobic organisms.

The pathogenic potential of *B. melaninogenicus* being manifested indirectly via the immune response of the host must also be considered. Recent studies on the immune response to oral *B. melaninogenicus* subsp. *asaccharolyticus*

strains showed that a high stimulation index to *B. melaninogenicus* subsp. *asaccharolyticus* antigens, using the in vitro lymphocyte blastogenesis assay, was obtained only in adults with advanced periodontal disease (14, 21). This, of course, correlates well with the findings that *B. melaninogenicus* subsp. *asaccharolyticus* is a prevalent organism in advanced periodontal lesions (23).

In summary, the present results indicate that *B. melaninogenicus* subsp. *asaccharolyticus* can play a central role in actively destructive periodontal disease. The above review of the pathogenic potential of *B. melaninogenicus* shows that several mechanisms exist whereby the periodontal tissues may be destroyed. Further studies are needed to characterize the molecular biology of the host response in periodontal disease and to develop a treatment for control of the number of *B. melaninogenicus* subsp. *asaccharolyticus* cells in the oral cavity and the periodontal pocket.

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