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Subgingival Microbiota in Squirrel Monkeys with Naturally Occurring Periodontal Diseases

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The squirrel monkey (*Saimiri sciureus*) has been proposed as an in vivo model for the study of subgingival colonization by suspected periodontopathogens, such as black-pigmented porphyromonads and prevotellas (BP/P). However, the indigenous microbiota of the squirrel monkey has not been well described. Therefore, in order to more fully characterize the oral microbiota of these animals, we studied two groups of squirrel monkeys from widely different sources. Group I consisted of 50 breeding colony monkeys ranging in age from 9 months to over 6 years which had been raised in captivity; group II consisted of 16 young sexually mature monkeys recently captured in the wild in Guyana. Group I animals in captivity had developed moderate to severe gingivitis, with a mean gingival index (GI) of 2.6; 52% of the sites bled, 26% had detectable calculus, and 83% had detectable BP/P. A group I subset (six animals), for which predominant cultivable microbiota was described, had a mean GI of 2.4. Colony morphology enumeration revealed that five of the six subset animals were detectably colonized with BP/P (range, 0 to 16.9%) and *Actinobacillus actinomycetemcomitans* (range, 0 to 3.9%); all subset animals were colonized with *Fusobacterium* species (range, 0.8 to 3.6%), *Actinomyces* species (range, 2.3 to 11%), and gram-positive cocci (range, 1.4 to 21.4%). Predominant cultivable microbiota results revealed the presence of many bacterial species commonly found in the human gingival sulcus. At baseline, group II animals were clinically healthy and had a mean GI of 1.4; 67% of the sites bled and 2.1% had calculus, and none of the animals had detectable BP/P. Neisseriae were very common in noninflamed sites. Subsequently, when inflamed sites were compared with noninflamed sites in group II animals after they had been maintained in captivity for 6 months, inflamed sites exhibited a more complex microbiota and increased proportions of gram-negative rods and asaccharolytic bacteria.

The squirrel monkey (*Saimiri sciureus*) has been used as an animal model for the study of the development and progression of periodontal diseases. Several studies have described the histological changes which occur following the placement of silk ligatures around the teeth of these animals (1, 5, 9, 19). Moreover, because of its humanlike dentition, ease of handling, and relatively low maintenance costs, the squirrel monkey may offer a practical model for the study of the bacterial etiology of periodontal disease. Rodent models, on the other hand, which have been used extensively to study periodontal disease, have a dentition and normal microbiota that differ markedly from those of humans.

Recently, studies have appeared in the literature describing the implantation of *Porphyromonas gingivalis* (formerly *Bacteroides gingivalis*) into nonhuman primates, including squirrel monkeys (8, 17). Evaluation of the effects of immunization on specific microorganisms which exist in the milieu of indigenous microbiota are complicated by microbial shifts and influences of the competing normal microbiota. While the normal and diseased oral microbiota of other monkeys, principally the macaques, have been described in some detail (11–13, 16, 22, 23), no description of the oral microbiota of squirrel monkeys is available. Without a knowledge of the indigenous healthy and diseased microbiota, correct inferences cannot be drawn on the potential microbial etiology of the periodontally diseased states in the squirrel monkey. Therefore, the purpose of this investigation was to more fully characterize the indigenous subgingival microbiota in the squirrel monkey and to compare the subgingival microbiota from inflamed and noninflamed sites.

MATERIALS AND METHODS

Animals. Two groups of squirrel monkeys were studied (Fig. 1). Group I consisted of 50 healthy animals from a large breeding colony at the University of South Alabama Medical Center, Mobile (3). They ranged in age from 9 months to over 6 years. All animals were in good health and were fed a diet of monkey chow (Ralston Purina Company, St. Louis, Mo.) and fresh fruit, and most were housed in family group runs consisting of one adult male and several females and juveniles. One cage housed only young males. At the time the animals were tested, they were not undergoing any other experimental use which would affect their subgingival microbiota. Two samples were taken from each monkey: samples from eight sites on the maxillary arch were pooled to constitute one sample, and samples from eight sites on the mandibular arch were pooled to constitute the second sample. From group I, a subset of six animals, with mean gingival indices (GIs) similar to the mean GI of the population and harboring subgingival black-pigmented porphyromonads and prevotellas (BP/P) ranging from 0 to 17% of the total cultivable microbiota, was selected to study the total cultivable microbiota of squirrel monkeys. The males and females were housed separately in two cages. Samples from all teeth were taken to evaluate the total microbiota.

Group II consisted of 16 sexually mature females (approximately 3 years old) which had been captured in their native habitat in Guyana and imported to the United States (South American Primates, Inc., Miami, Fla.) (3), where they were

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Group I Squirrel Monkeys (University of South Alabama)
(Table 1, 2,)
Clinical evaluation
Pooled Plaque Samples
ETSA
TSBV
A. Subset
(Table 4)
Clinical evaluation
1. (Table 1 and 5)
Whole Mouth Sample
Presumptive Identification
Colony morphology
GS
API 20A
2. (Table 3)(Fig. 1)
Sample Pooled from Upper and Lower Teeth
Predominant Cultivable Microbiota

Group II Squirrel Monkeys (Wild caught)
(Table 2)
Clinical Evaluation
(Table 5)
Pooled Plaque Samples
ETSA
TSBV
Mitis-salivarius
CFAT
MacConkey
A. Subset
(Table 4)
Clinical Evaluation
(Table 5)
Selective Media Presumptive Identification
B. Subset
(Tables 6 and 7) (Fig. 1)
Individual inflamed vs noninflamed sites
Predominant Cultivable Microbiota

FIG. 1. Monkey groupings and procedures. GS, Gram stain.

caged in large groups at the importation center. Animals were selected on the basis of good general and oral health, full dentition, sex, and similar approximate age (judged by an attending veterinarian). The animals were transported to the University of Florida Health Center Animal Resources Department, where they were housed in individual cages and maintained on a diet of water-softened Purina Monkey Chow no. 5058 (Ralston Purina) supplemented with fresh fruit and vegetables once a day. All monkeys were initially sampled within 4 to 6 weeks of arrival in a fashion similar to that employed in sampling the group I animals.

From group II, a subset of six animals with a mean GI similar to that of the entire group II population was selected to compare the levels of prominent taxa in this subset with those of the group I subset. Subsequently, 6 months later, a subset of two animals was selected to compare the microbiota associated with sites which had remained healthy with the microbiota associated with sites which had developed gingival inflammation over the previous 6 months. Four individual sites from each monkey were sampled (three inflamed [mean GI, 2.5 ± 0.5] and one noninflamed [mean GI, 1.0 ± 0]).

Microbial sampling, group I. To facilitate handling, clinical evaluation, and sampling, monkeys at the University of South Alabama (group I) were anesthetized with Ketamine (Parke, Davis & Co., Detroit, Mich.) at a concentration of 30 mg/kg of body weight. Gingival inflammation on the mesial interdental papillae of all teeth present was assessed on a range of 0 to 4 by using a noninvasive modification of the Löe and Silness index (4, 14). A GI of 2 or above was considered to denote inflammation.

Animals were also assessed for the presence of calculus, and the incidence of bleeding upon sampling with paper points was recorded at all sites sampled. The percentage of sampled sites which had visible calculus and which bled upon probing was calculated for each animal. Saliva and supragingival debris were removed with gauze, and microbial samples were taken by placing an extra fine endodontic paper point in the subgingival crevice and holding it in place for 10 s. To evaluate total microbiota, eight sites on each arch were sampled and pooled in tubes containing 4.5 ml of prereduced anaerobically sterilized Ringer's solution. After thorough vortexing, three 10-fold dilutions in prereduced anaerobically sterilized Ringer's solution were made under oxygen-free nitrogen gas (Liquid Air, San Francisco, Calif.). These samples were inoculated onto enriched tryptic soy blood agar (ETSA), as described by Syed et al. (25), and onto tryptic soy agar (Difco, Detroit, Mich.) supplemented with bacitracin (75 μg/ml) and vancomycin (5 μg/ml) (TSBV) for the enumeration of *Actinobacillus actinomycetemcomitans* (20).

All plates were transported back to the laboratory under anaerobic conditions generated by Gas Pak Anaerobic Systems (BBL, Cockeysville, Md.). TSBV plates were incubated in candle jars for 72 h at 37°C. ETSA plates were incubated for 7 days in an anaerobic atmosphere containing 5% CO_2, 10% H_2, and 85% N_2.

Total microbial counts per milliliter on ETSA plates were determined; certain taxa (BP/P, *Fusobacterium* spp., gram-positive cocci, *Actinomyces* sp., and surface translocating gram-negative rods) were enumerated on the basis of Gram stain, colony morphology, and catalase reaction, as described by Slots (21). Presumptive identification of colony types from major taxa was verified by API20A strips. All representative colony types were subsequently identified from a subset of six monkeys with average gingival inflammation in order to roughly quantitate indigenous bacteria cross-sectionally. Selected colonies were subcultured for purity onto ETSA and were frozen at −80°C in Todd-Hewitt broth supplemented with 2.5% dimethyl sulfoxide (Fisher Scientific Company, Orlando, Fla.).

Figure 2 depicts the scheme followed in the identification process of the bacterial isolates. Briefly, identification of gram-positive cocci was determined by oxygen tolerance, catalase production, and reaction to biochemical tests as determined by either the API20A (anaerobic) or the API20S (facultative streptococci) systems (Analytab Products, Plainview, N.Y.).

Gram-negative cocci were identified by oxygen tolerance and catalase and oxidase production. Oxygen-tolerant, catalase-positive, oxidase-positive organisms were identified with the Minitek system for neisseriae (BBL). Oxygen-sensitive gram-negative cocci were identified with the API20A system.

Gram-positive rods were identified by spore production, motility, reaction to biochemicals in the API20A system, and gas-liquid chromatographic end product analysis using peptone yeast glucose media (7).

Facultative gram-negative rods were identified by their growth patterns on MacConkey agar (BBL) by using the API20E (enteric gram-negative rods) and/or the Minitek

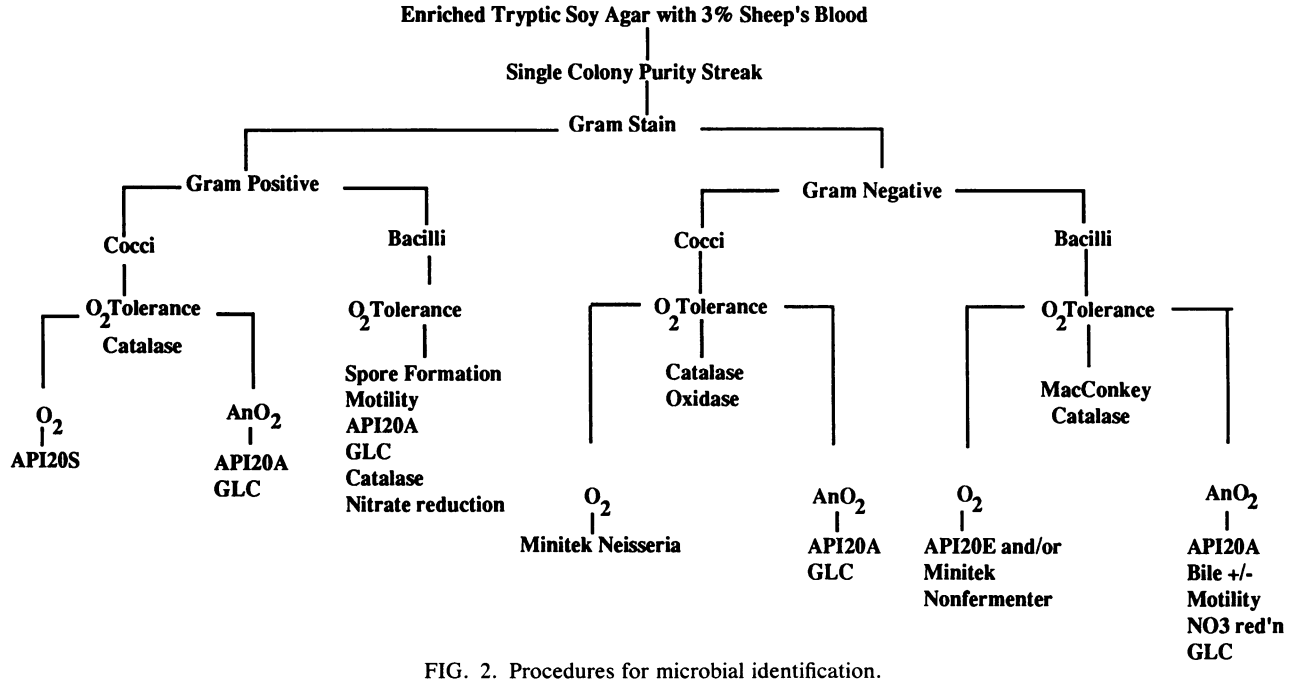

FIG. 2. Procedures for microbial identification.

nonfermenter (BBL) system. The addition of traditional growth enhancers such as serum, Tween 80, sodium formate, and sodium fumerate proved to be inconclusive.

Anaerobic gram-negative rods were identified by the API20A system plus gas chromatographic end product analysis, bile tolerance, motility, and reduction of nitrate. A flow chart of the identification protocol is shown in Fig. 2.

Microbial sampling, group II. The monkeys caught in the wild (wild-caught monkeys; group II) were handled and sampled as described above, except that in addition to ETSA and TSBV, the following selective media were used: CFAT (26), for the enumeration of *Actinomyces* species, and mitis-salivarius (Difco), for the enumeration of streptococci. Since these animals were sampled at the University of Florida animal facility, the plates were immediately transferred to an anaerobic chamber upon inoculation of the sample. Estimates of the percentages of streptococci on mitis-salivarius agar versus total counts on the ETSA were determined. In a similar manner, the estimated percentages of *Actinomyces* organisms on CFAT agar and of *A. actinomycetemcomitans* on TSBV were calculated. Percentages of BP/P, *Fusobacterium* spp., and spreading colonies were calculated directly from ETSA.

A subset of two wild-caught monkeys was sampled after they had spent 6 months in captivity; as mentioned in the previous section, only four sites per monkey were sampled separately (three inflamed and one noninflamed) to compare microbiota associated with diseased sites and healthy sites.

Bacteria were enumerated by colony morphology on ETSA, and each representative colony type was subcultured and identified as described in the legend to Fig. 2.

RESULTS

To detect correlations in the group I monkeys, a Pearson correlation test was run on the increasing age and GI relative to the presence of certain clinical and bacteriological characteristics. The results of this analysis are shown in Table 1.

Clearly, there is a highly significant relationship between increasing age and increasing GI, the presence of calculus, the increasing percentage of BP/P, and the increase of the total number of cultivable bacteria. Similar relationships have been noted in studies of human subjects (2, 10, 15, 18). Conversely, there was no statistical relationship between age and the presence of surface translocating gram-negative rods, *Fusobacterium* spp., or *A. actinomycetemcomitans*.

Similarly, strong correlations between increasing GI and the presence of calculus and bleeding and the increase of total cultivable microbiota were found. The correlation between the percentage of BP/P and the GI was also significant ($P = 0.07$). No other bacterial group examined exhibited a statistically significant correlation with GI.

Table 2 lists the clinical evaluations of all the group I and group II animals and the subsets of six animals within these groups which were examined more closely. Within the group I subset, the mean GI, the percentage of sites with calculus,

TABLE 1. Mean values of clinical and bacteriological characteristics of group I (breeding colony) monkeys and significance of age relatedness

Variable[a]	Mean ± SD (n = 100)	Age-related P value	GI-related P value
Age (yr)	4.9 ± 2.2	0.0000	0.001
GI	2.5 ± 0.3	0.001	0.0000
Calculus[b]	0.45 ± 0.38	0.0002	0.0001
Sites which bled[b]	0.92 ± 0.14	0.51	0.0001
TCM (CFU/ml)	$1.1 \times 10^8 \pm 7.9 \times 10^7$	0.002	0.0001
% BP/P	4.1 ± 6.3	0.0005	0.07
% Spreaders	1.6 ± 3.6	0.382	0.650
% Fuso	4.0 ± 7.8	0.200	0.497
% Aa	0.03 ± 0.1	0.256	0.598

[a] Abbreviations: Fuso, fusobacteria; Aa, *A. actinomycetemcomitans*; TCM, total cultivable microbiota.

[b] Numerically tabulated so that 1 denotes presence of calculus or bleeding and 0 denotes its absence.

TABLE 2. Summary of selected characteristics of group I and group II squirrel monkeys

Group	GI (mean ± SD)	% Sites bleeding	% Sites with calculus	% Animals infected with BP/P
Group I				
Total population (n = 51)	2.5 ± 0.6	53	26	82.6
Subset (n = 6)[a]	2.4 ± 0.1	91.6	20.9	83.3
Group II				
Total population (n = 16)	1.4 ± 0.2	67	2.1	0
Subset (n = 6)	1.4 ± 0.1	65.4	1.6	0

[a] Five subset animals had moderate gingival inflammation and were colonized with BP/P. One subset animal had a similar degree of gingival inflammation but was not colonized with BP/P.

and the percentage of animals orally infected with BP/P were similar to those statistics for the group at large. The group I subset differed noticeably only in the number of sites which bled upon probing. Overall, the subset animals were selected to represent an average cross-section of the population. The clinical characteristics of all the group I animals compared with those of all the group II animals, however, are more striking in their differences. BP/P were undetectable, and the GI and calculus scores were much lower.

Table 3 lists the pooled predominant subgingival microbiota from the group I subset (six monkeys). All representative colony types were picked for identification; 12.2% were gram-positive cocci, 22.2% were gram-positive rods, 6.1% were gram-negative cocci, and 49.5% were gram-negative rods. Specifically, streptococci predominated among the gram-positive cocci and bacilli and *Actinomyces* sp. predominated among the gram-positive rods. Neisseriae were the only gram-negative cocci.

Over 16% of the total gram-negative rods were identified as BP/P, the largest group proportionally of any bacterial genus (Table 3). There were also relatively high proportions of surface translocating gram-negative rods, *Alcaligenes* sp., CDC group II organisms (whose characteristics are listed in Table 3), and *Fusobacterium* sp. Interestingly, nearly all these predominantly gram-negative rods are nonfermentative organisms.

Table 4 compares selected clinical parameters of the subset of six group I monkeys maintained in captivity with those of a subset of six representative individuals from the group II monkeys recently captured in the wild. All the monkeys were sexually mature (≥3 years of age), and yet the GI of the wild-caught monkeys is significantly ($P < 0.0001$) lower than that of the captive monkeys. The differences in GI between wild-caught and captive monkeys did not seem to be attributable to the slight difference between the known age of the captive monkeys and the presumed age of the wild-caught monkeys, since even captive monkeys under one year of age had a GI of >2.0 (3). Captivity seemed to promote gingival inflammation.

In the six captive monkeys, the mean GI of sites actually sampled varied only slightly from the mean GI of all sites evaluated. In the six wild-caught monkeys, the mean GI of the sites sampled was slightly higher than the mean for all sites evaluated in all 16 wild-caught monkeys.

Table 5 lists the levels of predominant genera found in the two squirrel monkey subsets that are also frequently encountered in human subgingival plaque samples. The mean GI of each monkey is shown for reference. The percentages of

TABLE 3. Predominant subgingival microbiota of squirrel monkeys

Organisms	% of total microbiota
Gram-positive cocci	
Beta-hemolytic cocci	0.9
Streptococcus sp.	6.1
Staphylococcus sp.	2.0
S. intermedius	0.9
Unidentified	2.3
Total	12.2
Gram-positive rods	
Bacillus sp.	12.7
Actinomyces sp.	6.3
Clostridium sp.	0.5
Eubacterium lentum	0.9
Lactobacillus fermentum	0.7
Unidentified	1.1
Total	22.2
Gram-negative cocci (*Neisseria* sp.)	6.1
Gram-negative rods	
Alcaligenes sp.	5.0
BP/P	16.5
Campylobacter sp.	0.5
Cardiobacterium-like[a]	2.9
CDC group IIj[b]	4.1
Fusobacterium sp.	3.8
A. actinomycetemcomitans	1.1
A. haemolyticus	0.7
Klebsiella sp.	2.0
Nonpigmented *Bacteroides* sp.	2.9
Vibrio sp.	2.0
Surface translocating rods (unidentified)	7.9
Total	49.5
Organisms lost	10.0
Total organisms	100.0

[a] Catalase-negative, oxidase-positive, nitrate-negative, indole-positive anaerobic rods.
[b] Catalase-positive, oxidase-positive, asaccharolytic facultative rods.

streptococci and actinomycetes were calculated from nonselective media for group I and from selective media for group II. These are not results of predominant microbiota studies and represent estimated percentages only. Furthermore, the values may not be comparable, since selective media often tend to enhance the expression of the target organism. Perhaps most significantly, BP/P were undetectable in the wild-caught monkeys but were common in the captive monkeys.

The percentages of all other selected genera except fusobacteria were higher in the captive than in the wild-caught monkeys, and yet the raw numbers of total bacteria per milliliter recovered from the pooled sites were not remarkably different.

Inflamed versus noninflamed sites. Results of the clinical evaluation revealed a nearly twofold difference between the mean GI of group I animals and that of group II animals. The data shown in Table 5 suggest that differences in the degree of gingival inflammation may be associated with differences in the subgingival microbiota. However, because the technique used in cultivating plaque samples from group I animals was slightly different from that used in cultivating plaque samples from group II animals, we wanted to com-

TABLE 4. Comparison of clinical characteristics of two populations of squirrel monkeys

Group and no. of monkey	Mean GI ± SD		Sex	Age (yr)	Wt (g)
	All sites[a]	Sampled sites			
Group I[c]					
1040	2.4 ± 0.5	2.5 ± 0.53	M	4	833
1052	2.3 ± 0.48	2.2 ± 0.46	M	4	834
1104	2.5 ± 0.51	2.7 ± 0.46	M	3	720
975	2.3 ± 0.46	2.0 ± 0	F	4	648
990	2.3 ± 0.49	2.7 ± 0.46	F	4	688
993	2.5 ± 0.51	2.9 ± 0.35	F	4	586
Total	2.4 ± 0.1	2.5 ± 0.31	NA[b]	NA	NA
Group II[d]					
1	1.3 ± 0.4	1.6 ± 0.5	F	3	527
6	1.4 ± 0.5	1.7 ± 0.4	F	3	572
7	1.3 ± 0.5	1.5 ± 0.5	F	3	598
8	1.5 ± 0.5	1.8 ± 0.4	F	3	525
11	1.5 ± 0.5	1.6 ± 0.5	F	3	490
18	1.7 ± 0.5	1.9 ± 0.2	F	3	497
Total	1.45 ± 0.1	1.7 ± 0.2	NA	NA	NA

[a] The GI used had a range of 0 to 4 (4).
[b] NA, not applicable.
[c] Animals from breeding colony.
[d] Animals caught in the wild.

pare the differences in the microbiota associated with inflamed and noninflamed sites by using identical cultural techniques. Approximately 6 months after the initial sampling presented in Table 5, we selected three sites from two monkeys in group II which had become inflamed during the time since the initial evaluation. Sites which had remained noninflamed were also selected.

Tables 6 and 7 present comparisons of the pooled data on the microbiota recovered from noninflamed and inflamed sites of group II monkeys. The microbiota of the noninflamed sites appeared to be less complex than that of the inflamed sites. Gram-positive and gram-negative cocci predominated in the noninflamed sites, and the proportions of *Neisseria* sp. were surprisingly high relative to their levels in humans (24). This observation corroborates earlier unpublished data from this laboratory. The composite microbiota of the inflamed sites is considerably more diverse (Table 7). The percentage of gram-positive cocci is nearly a third that found in the noninflamed sites. Fewer presumptive *Neisseria* organisms were detected, and instead the microbiota had shifted to a predominantly gram-negative population, which accounted for 57% of the colony types recovered from inflamed sites. Members of the genus *Fusobacterium* were common in this subpopulation. Nonfermentative organisms which fit no known classification were assigned to Periodontal Disease Research Center (PDRC) groups, the characteristics of which are listed in Table 7. These types of organisms were

TABLE 6. Predominant subgingival microbiota found in noninflamed subgingival sites[a]

Organisms[b]	% of total microbiota
Gram-positive cocci	
Staphylococcus sp.	21.5
Streptococcus sp.	1.0
Total	22.5
Gram-positive rods	
Bacillus sp.	8.9
Actinomyces naeslundii	7.9
Total	16.8
Gram-negative cocci (*Neisseria* sp.)	42.4
Gram-negative rods	
Serratia rubidaea	4.7
Fusobacterium sp.	13.6
Total	18.3
Total organisms	100.0

[a] Data from two noninflamed sites, one in each of two monkeys.
[b] The average number of CFU per milliliter is $4.1 \times 10^6 \pm 5.6 \times 10^6$.

TABLE 5. Comparison of prominent members of microbiota in the gingival crevices of two populations of squirrel monkeys

Group and no. of monkey	Mean GI[a]	% of total microbiota						Total flora/ml
		Gram-positive cocci	Actinomycetes	BP/P	Fusobacteria	Surface translocating rods	*A. actinomycetemcomitans*	
Group I								
1040	2.4	21.4	4.9	0.0	0.8	0.02	0.3	5.6×10^7
1052	2.3	5.8	5.8	1.0	1.2	1.0	1.5	4.3×10^7
1104	2.5	8.6	2.6	1.6	0.6	0.08	3.9	1.5×10^8
975	2.3	1.4	8.1	2.4	3.4	4.5	1.3	6.9×10^7
990	2.4	2.8	11.0	15.5	3.6	1.4	0.0	6.8×10^7
993	2.6	3.7	2.3	16.9	2.4	14.4	0.1	1.6×10^8
Total	2.4 ± 0.1	7.3 ± 7.4	5.8 ± 3.3	6.2 ± 7.8	2.0 ± 1.3	3.6 ± 5.5	1.2 ± 1.5	$9.1 \times 10^7 \pm 5 \times 10^7$
Group II								
1	1.3	24.6	8.5	0.0	5.8	0.8	0.01	5.7×10^7
6	1.4	1.7	0.1	0.0	13.3	0.3	0.0	7.8×10^7
7	1.3	19.8	0.4	0.0	2.4	0.0	0.0	1.2×10^8
8	1.5	19.3	8.0	0.0	7.1	1.6	0.0	5.8×10^7
11	1.5	28.4	0.3	0.0	5.1	7.4	0.0	2.3×10^7
18	1.7	4.3	0.2	0.0	15.0	0.8	1.2	4.9×10^7
Total	1.45 ± 0.1	16.4 ± 10.9	2.92 ± 4.1	0.0	8.12 ± 4.9	1.82 ± 2.8	0.2 ± 0.5	$6.3 \times 10^7 \pm 3.3 \times 10^7$

[a] Mean GI of sites sampled.

TABLE 7. Predominant subgingival microbiota found in inflamed subgingival sites[a]

Organisms[b]	% of total microbiota
Gram-positive cocci	
Streptococcus sp.	7.4
Staphylococcus sp.	0.9
Peptococcus sp.	0.2
Total	8.5
Gram-positive rods	
Arachnia sp.	1.3
Unidentified rods	1.9
Actinomyces sp.	8.5
Propionibacterium acidi-propionici	1.7
Eubacterium lentum	1.3
Total	14.7
Gram-negative cocci (*Neisseria* sp.)	6.2
Gram-negative rods	
PDRC group I[c]	6.0
A. actinomycetemcomitans	5.3
PDRC group II	4.6
PDRC group III	2.5
Moraxella sp.	1.9
Enterobacterium agglomerase	0.9
Campylobacter sp.	0.2
Capnocytophaga sp.	0.2
Fusobacterium sp.	24.0
Cardiobacterium-like	3.2
Bacteroides sp.	2.8
Vibrio sp.	0.2
Total	51.8
Unidentified organisms	18.7
Total organisms	100.0

[a] Data from three inflamed sites in two monkeys.
[b] The average number of TCF per milliliter is $2.5 \times 10^7 \pm 2 \times 10^7$.
[c] PDRC group I: anaerobic gram-negative rods, oxidase positive, catalase negative, asaccharolytic, gel positive, indole negative, nitrate reducing; PDRC group II: facultative gram-negative rods, oxidase positive, catalase positive or negative, asaccharolytic, gel positive or negative, indole negative, nitrate reducing; PDRC group III: facultative gram-negative rods, oxidase negative, catalase negative, saccharolytic, gel positive or negative, indole negative, nitrate reducing.

encountered frequently. *A. actinomycetemcomitans* was also detected in the inflamed sites in relatively high numbers. However, no BP/P were detected in these sites.

We recovered a number of *Cardiobacterium*-like organisms which had the characteristic "striped" cellular morphology and which produced catalase, indole, and variable sugar fermentation patterns.

Most striking overall were the differences in degrees of complexity of microbiota between the inflamed and the noninflamed sites. In addition, the average numbers of bacteria recovered from inflamed sites were over six times higher than those recovered from noninflamed sites (2.5×10^7 versus 4.1×10^6).

DISCUSSION

Prior to the 1970s, efforts to establish etiologies and mechanisms of experimentally induced periodontal disease were based on rodent models, principally because of their availability, ease in handling, and economy. However, significant dissimilarities in dentition and oral microbiota make these animals less suitable in applications relating to human periodontal disease. In 1970, researchers began to examine nonhuman primates, such as squirrel monkeys, as models for experimental periodontal disease. More recently, we have examined the normal microbiota of captive squirrel monkeys, since these animals are known to spontaneously develop periodontal disease under caged conditions and to possess a dentition similar to that of humans (3, 9). Our laboratory is using this model to study the effects of immunization on implantation or emergence of known periodontopathogens. We have also detected BP/P, specifically *Porphyromonas gingivalis* and *Prevotella intermedia*, in the microbiota of captive squirrel monkeys, maintained at the University of Florida Health Center Animal Resource Department, which had developed clinically detectable, naturally occurring periodontitis over a prolonged period of captivity (>5 years; unpublished observations). These observations and corroborative work published by other investigators (10, 16, 22, 23) demonstrating the presence of BP/P in other nonhuman primates supported a more detailed examination of the indigenous oral microbiota of the squirrel monkey.

The primary purpose of this study was to describe the indigenous subgingival microbiota of squirrel monkeys. In surveying the subgingival microbiota of group I animals (mean GI = 2.5) and group II animals (mean GI = 1.4), plaque samples were pooled to increase the probability of detecting a particular taxon if it was present. For example, if BP/P were present in an animal with a low GI, pooling plaque from all maxillary teeth or all mandibular teeth maximized the chance of detecting them.

In the initial survey of group I monkeys, we presumptively grouped bacteria from 50 animals (3) on the basis of selective media and distinctive colony morphology. Using these groupings, we detected statistically significant associations between the presence of BP/P and the animal's age ($P < 0.0005$) and the mean GI ($P < 0.07$) of the animal's oral cavity. There is a similar correlation between BP/P and age as well as GI in humans (2, 10, 15, 18). Also, the total number of bacteria cultivable from the periodontal pockets increased in direct relationship to age and GI, and it was highly significant in each case ($P < 0.002$ and < 0.0001, respectively).

No significant correlation between any of the other bacteria presumptively identified and the GI or the age of the animals was noticed.

When colony types were identified within a subset of six group I monkeys, the pooled microbiota was composed of nearly 50% gram-negative rods, which were largely BP/P (Table 3). There are many similarities between the representative genera found in these monkeys and those found in the human oral cavity, notably representatives of presumptive streptococci, actinomycetes, *Fusobacterium* sp., *Eubacterium* sp., *Campylobacter* sp., surface translocating gram-negative rods, and *A. actinomycetemcomitans*. The presence of the latter species in both inflamed and noninflamed sites suggests it may be a relatively benign colonizer in squirrel monkeys, since it does not seem to correlate with inflammation or disease.

The average GI of the group I monkeys was 2.4 ± 0.1, somewhat higher than that of six wild-caught monkeys in group II that were sampled (Table 4). All animals compared in Table 4 were sexually mature. The age of the wild-caught monkeys was estimated to be approximately 3 years. All 3- to 4-year-old captive monkeys ($n = 27$) from group I had an average GI of 2.5 ± 0.3 (3), compared with an average GI of

1.4 ± 0.1 for ≥3-year-old wild-caught monkeys. Interestingly, in group I, even the monkeys under one year of age (n = 8) had a mean GI of 2.2 ± 0.2 (3), suggesting that the captive conditions or diet may discourage natural hygiene, leading to the development of oral pathology.

Although the wild-caught monkeys had no detectable BP/P (Table 5), 84% of the captive monkeys had detectable BP/P, with percentages ranging to nearly 29%. The association between the presence of BP/P and increasing GI was close to statistical significance ($P < 0.07$), and there was a highly significant association between the presence of BP/P and increasing age ($P < 0.0005$).

BP/P were not detected in monkey no. 1040. However, we have previously observed that each of the six animals in the group I subset, including monkey 1040, had significantly higher levels of anti-BP/P immunoglobulin G in serum than did group II animals (3, 17), suggesting that these animals were or had been colonized by BP/P. In contrast, BP/P were not detected in any group II animals, and the low anti-BP/P immunoglobulin G levels in serum are consistent with the absence of detectable BP/P (3, 17). This suggests that these animals had never been colonized with BP/P. It is of interest that monkey 1040, which appeared to be devoid of BP/P, also harbored nearly three times more gram-positive cocci than the mean percentage of gram-positive cocci for the subset of group I monkeys (Table 5). Similarly, no BP/P were detected in the wild-caught group II monkeys, and the mean level of gram-positive cocci in this group was more than twice the mean level in the captive group I animals. Hillman and coworkers have shown that certain streptococcal strains can inhibit periodontal pathogens such as *P. gingivalis* and *P. intermedia* (6). Although we did not investigate this possibility in the present study, the squirrel monkey model might be useful for investigating bacterial inhibition of putative periodontal disease-associated bacteria such as BP/P.

Although in the captive monkeys there was a distinct age-related correlation with the levels of BP/P in the gingival sulcus, the wild-caught monkeys at a similar age did not harbor detectable BP/P. Once again, captivity seemed to predispose the monkeys to infection with BP/P. The precise reason for this is unclear, and a number of contributing factors are possible. Dietary and diet-related abrasive actions on plaque microorganisms may play some role (unpublished observations). Other possibilities contributing to this phenomenon might include stress, human contact, time, etc.

The wild-caught monkeys provided us the opportunity to sample nondiseased sites. After six months in captivity, most animals developed inflammation at some sites, providing an opportunity to compare the microbiota from inflamed and noninflamed sites in the same animal and cultured under the same conditions. Two animals were selected and sampled for predominant cultivable microbiota in pooled noninflamed and pooled inflamed sites. Although the data presented here represent a small data set and therefore are not definitive, they are generally consistent with data shown in Tables 3 and 5, which were obtained from the larger data set. The results of this procedure suggest that as inflammation develops, bacteria appear which are more comparable to human periodontal disease-associated bacteria (*Fusobacterium* sp., *Actinobacillus* sp., *Capnocytophaga* sp.) and a shift occurs from simple to complex, from gram-positive to gram-negative and from saccharolytic to asaccharolytic microbiota. We can only speculate that BP/P would have emerged in these animals if they had been sampled later.

However, in noninflamed sites, a large proportion of *Neisseria* species were encountered which do not parallel normal oral microbiota from the healthy human periodontium. The number of streptococci and the number of actinomycetes were lower than would be expected from nondiseased sites in humans.

Of primary importance in this study, however, is the suitability of the squirrel monkey as a model for periodontal disease research. Several similarities between the subgingival microbiota in squirrel monkeys and in humans were observed. As presented in the total microbiota survey, the monkeys are indeed colonized by a number of bacterial genera associated with human periodontal health and disease. The microbiota changes in complexity and shifts from a gram-positive to a gram-negative profile as inflammation increases. BP/P (*P. gingivalis* and *P. intermedia*) are also associated with increased inflammation and are readily cultivated from the periodontal pockets of captive animals. There are some minor discrepancies in microbiota (e.g., the prevalence of *Neisseria* sp. in healthy sites and the abundance of nontypable, nonfermenting gram-negative rods in diseased sites), but overall the similarities are striking and justify the continued use of this animal model for the study of periodontal disease.

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