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Microbial Ecology of Plaque in Rats with Naturally Occurring Gingivitis

EMIKO ISOGAI, HIROSHI ISOGAI, HIROKO SAWADA, HISAYUKI KANEKO, AND NOBUYOSHI ITO*

Department of Preventive Dentistry, School of Dentistry, Higashi Nippon Gakuen University, Ishikari-Tobetsu 061-02, Japan

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The microbial ecology of adherent plaque was investigated in relation to the pathological findings of gingivitis in plaque-susceptible rats. Plaque developed in the gingiva of the lower incisor in plaque-susceptible rats, but not in plaque-resistant rats, after they were fed a commercial powder diet. With increase in plaque volume, the total counts of bacteria increased 10^9 to 10^{11}/g. In the first 3 months, *Bacteroides* species increased and became the predominant population. *Streptococcus* species also increased at the same time. After 9 months, *Fusobacterium* species and oral *Treponema* species were recognized in increasing numbers. The anaerobic bacteria increased in proportion with the progression of plaque development. *Bacteroides intermedius*, *Fusobacterium nucleatum*, *Streptococcus salivarius*, and other species were isolated. Acute gingivitis was observed within 3 months, and subacute-chronic gingivitis was observed between 2 and 12 months. These findings suggest that proportional changes in the gingival plaque flora may uniquely contribute to the development of gingival inflammation in this experimental model.

Bacterial plaque has been associated with periodontal disease on the basis of epidemiological (25) and clinical (11, 14) observations. Human periodontal disease is associated with a complex microflora, in which more than 250 bacterial species can be encounted. Recent studies show that the proportion of gram-negative anaerobic organisms increases markedly in the subgingival microflora with increasing severity of periodontal disease (12, 19, 20, 23, 28). Studies of specific gram-negative bacteria which play a major role in the etiology of periodontal disease have been reported (4, 22). So far, very limited studies have been done to correlate bacterial profile with the development of gingivitis by using an appropriate animal model with naturally developing periodontal diseases. Plaque-susceptible (SUS) and plaque-resistant (RES) rats were derived from the Wistar Kyoto strain. SUS rats show remarkable plaque formation and naturally occurring gingivitis, but RES rats do not (1, 2, 8–10). This study is concerned with bacteriological and histopathological examinations of developing gingivitis in SUS rats.

MATERIALS AND METHODS

Animals. SUS and RES rats (1, 8, 9) were used for experiments. Both strains were derived from the Wistar Kyoto strain. At present, the 20th and later generations of these partially inbred strains are available. Both strains were fed a commercial powder diet and examined at 1, 3, 6, 9, and 12 months. More than 150 SUS and 80 RES rats were used for experiments.

Contact infection and plaque inoculation. Ten RES rats were raised with ten SUS rats from the ages of 5 weeks to 3 months. Existence of contact infection was judged by plaque accumulation and gingival index. Plaque inoculation was done on a total of 25 RES rats (5 weeks old and 1, 3, 6, 9 months old) from the same-age SUS rats. An amount of 1 to 10 mg obtained from SUS rats was inoculated on the gingival surfaces of RES rats twice a week for 3 months. After 3 days of plaque inoculation, plaque accumulation and gingival inflammation were examined twice a week.

Collection of plaque. Plaque was collected from some lower incisors of SUS rats under a stream of oxygen-free CO_2 gas. Each plaque sample was weighed and immediately placed in sterile oxygen-free tubes with GAM broth (Nissui Co.).

Bacteriological procedures. Each plaque sample was dispersed by 10 to 15 s of sonic oscillation under a stream of oxygen-free CO_2 gas. The suspension was placed an anaerobic glove box (Hirasawa Co.) with 80% N_2–10% H_2–10% CO_2 and serially diluted, and 0.1 ml was plated on each agar plate containing the following nonselective media for anaerobes: modified Egger-Gagnon (EG) agar (18) ([per 1,000 ml] horse blood, 70 ml; proteose peptone, 10.0 g; yeast extract, 5.0 g; L-cysteine, 0.5 g; glucose, 1.5 g; potassium hydrogen phosphate, 4.0 g; starch, 0.5 g; L-cysteine monohydrochloride, 0.5 g; silicon, 0.2 g; Tween 80, 0.5 g; hemin, 0.003 g; agar, 15.0 g; beef extract, 930 ml), TF agar (Nissui Co.) ([per 1,000 ml] horse blood, 70 ml; Trypticase, 17.0 g; proteose peptone, 10.0 g; yeast extract, 3.0 g; L-cysteine, 0.25 g; sodium sulfite, 0.1 g; sodium thioglycolate, 0.5 g; sodium chloride, 7.5 g; glucose, 6.0 g; lactose, 5.0 g; phiton, 3.0 g; hemin, 0.003 g; agar, 15.7 g; heart infusion, 500 ml), GAM agar (Nissui Co.) ([per 1,000 ml] horse blood, 70 ml; proteose peptone, 10.0 g; yeast extract, 5.0 g; sodium thioglycolate, 0.3 g; sodium chloride, 3.0 g; starch, 5.0 g; L-cysteine monohydrochloride, 0.5 g; peptone, 10.0 g; peptone soya, 3.0 g; digested serum, 13.5 g; beef extract, 2.2 g; liver extract, 1.2 g; potassium dihydrogen phosphate, 2.5 g; hemin, 0.003 g; agar, 15.0 g), Trypticase soy blood (TS) agar (BBL Microbiology Systems), with 5% horse blood. Samples also were plated on NBGT agar (16) (EG agar, 1,000 ml; sodium taurocholate, 2.5 g; brilliant green, 0.0025 g; neomycin, 0.5 g), *Bacteroides* selective agar (Nissui Co.) and *Bacteroides* agar (30) for *Bacteroides* species, modified FM agar for *Fusobacterium* species (Nissui Co.) ([per 1,000 ml] yeast extract, 10.0 g; sodium thioglycolate, 0.3 g; sodium chloride, 3.0 g; glucose, 3.0 g; starch, 5.0 g; L-cysteine monohydrochloride, 0.3 g; agar, 14.7 g; peptone, 20.0 g;

* Corresponding author.

TABLE 1. Pregingivitis flora of SUS and RES rats

Bacteria	% Prominent oral flora in:							
	Saliva		Tongue dorsum		Buccal mucosa		Gingival crevice	
	SUS	RES	SUS	RES	SUS	RES	SUS	RES
Gram-negative rods								
Bacteroides spp.	0	0.6	0.5	0.4	0.1	0	1.4	1.7
Fusobacterium spp.	0.6	0	0.2	0	0.3	0	0.2	<0.1
Enterobacteriaceae	5.2	5.3	4.0	6.5	9.1	4.1	5.6	5.7
Others	1.1	0.7	2.5	2.6	3.1	4.1	10.2	5.5
Gram-positive cocci								
Streptococcus spp.	67.9	71.0	68.9	72.5	56.1	66.2	59.9	56.0
S. salivalius	33.3	39.6	20.3	34.4	26.3	32.7	18.1	28.6
S. mitis	15.0	17.9	36.6	23.4	22.2	21.9	10.4	9.4
S. sanguis	14.7	10.3	12.4	15.0	14.2	11.4	24.0	18.0
S. mutans	<0.1	0	0	0	0	0	3.5	1.3
Staphylococcus spp.	0.3	0.7	0.1	8.5	1.2	2.5	0	1.3
Others	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Gram-positive rods								
Lactobacillus spp.	9.5	12.4	9.8	7.4	11.8	3.3	9.1	8.5
Others	0.2	1.5	0.6	0.2	1.1	0.6	0.2	0.3
Gram-negative cocci								
Veillonella spp.	6.5	2.0	7.6	3.0	9.2	5.5	7.3	10.7
Neisseria spp.	8.8	5.6	5.7	6.7	7.9	13.7	5.8	8.1
Others	<0.1	<0.1	<0.1	<0.1	0.1	<0.1	<0.1	<0.1

peptone soya, 1.5 g; digested serum, 6.75 g; beef extract, 1.15 g; liver extract, 0.6 g; potassium dihydrogen phosphate, 2.5 g; whale meat extract, 5.0 g; neomycin, 0.2 g; crystal violet, 0.01 g), Mitis Salivarius (MS) agar for *Streptococcus* species (Difco Laboratories), MS agar with 20% sucrose and 0.2 U of bacitracin per ml (MSB agar) for *Streptococcus mutans*, brain heart infusion agar for gram-positive rods, Rogosa selective *Lactobacillus* agar (Difco) and *Lactobacillus* selective agar (Nissui Co.) for *Lactobacillus* species, Sabouraud glucose agar for molds (Difco), DHL agar for members of the family *Enterobacteriaceae* (Eiken Co.), and modified VS agar for *Veillonella* species (Difco). Each plate was incubated at 37°C for 3 to 7 days in the anaerobic glove box. After incubation, the number of colonies of each recognizable type was counted. Representative colonies on nonselective media and one colony of each type on selective media were picked and colonized two or three times. Each strain from anaerobic culture media was subcultured on EG agar to distinguish obligate from facultative anaerobes. All strains were Gram stained and classified into genera or families on the basis of morphological and cultural characteristics.

Identification of representative colonies was done mainly by the Minitek system (BBL) (24, 29). The count of each bacterial group, per gram of wet material, was calculated from the number of colonies in suitable dilutions with each culture medium. When the count in the nonselective media was higher than that in the selective media, the former was regarded as the accurate viable count of the corresponding bacterial group. The total viable count was calculated from the sum of the counts of each bacterial group. Identification of representative colonies was done mainly by the Minitek system.

Immunodiffusion. Immunodiffusion was used for identification of *Bacteroides* species. The agar gel for immunodiffusion was prepared with distilled water at a concentration of 1% agar.

Immune sera were obtained from rabbits hyperimmunized with *B. intermedius* NCTC 9336, *B. melaninogenicus* ATCC 25845, *B. levii* VPI 10450, *B. gingivalis* 381, *B. gingivalis* ATCC 33277, *B. oralis* VPI 7570, or *B. loescheii* ATCC 15930. Each strain was injected seven times intravenously into one rabbit at intervals of 4 to 7 days with increasing amounts of Formalin-killed bacterial cells (ca. 10^7 to 10^9 cells). Anti-*B. gingivalis* lipopolysaccharide (B-LPS) antiserum was also used. B-LPS was extracted from *B. gingivalis* ATCC 33277 by the phenol-water method (27), purified by RNase treatment and ultracentrifugation, and then injected into a rabbit intracutaneously with Freund complete adjuvant. Anti B-LPS antiserum was species specific (data not shown). Antigens also were prepared from *Bacteroides* isolates from plaque. Cells of each strain grown in 10 ml of GAM broth were collected and suspended in 1 ml of phosphate-buffered saline (0.01 M [pH 7.2]). An equal volume of 1% sodium dodecyl sulfate (SDS) was added, the mixture was incubated at 37°C for 1 h and then clarified by centrifugation at 2,000 × *g* for 15 min, and the supernatant was used as antigen.

Counts of spirochetes. Samples were diluted to a final concentration of 1 mg of plaque per ml. About 5 μl was placed on a glass slide, and the number of spirochetes per field was counted by using a dark-field microscope (×200).

Plaque volume and gingival index. Each plaque sample was obtained from the surfaces and pockets of the lower incisors and weighed directly. The degree of gingivitis about the lower incisors was estimated by a modification (10) of the gingival index (13).

Briefly, criteria for the gingival index system were as follows. The index for acute inflammation (counter area in the gingiva) was 0, absence of inflammation; 1, mild inflammation with slight change to reddish color and a little swelling; 2, moderate inflammation with change in reddish color, obvious swelling, and glazing and bleeding on pressure; and 3, severe inflammation with change in reddish color, pronounced swelling, and spontaneous bleeding. The index for chronic inflammation (extensive area in the gin-

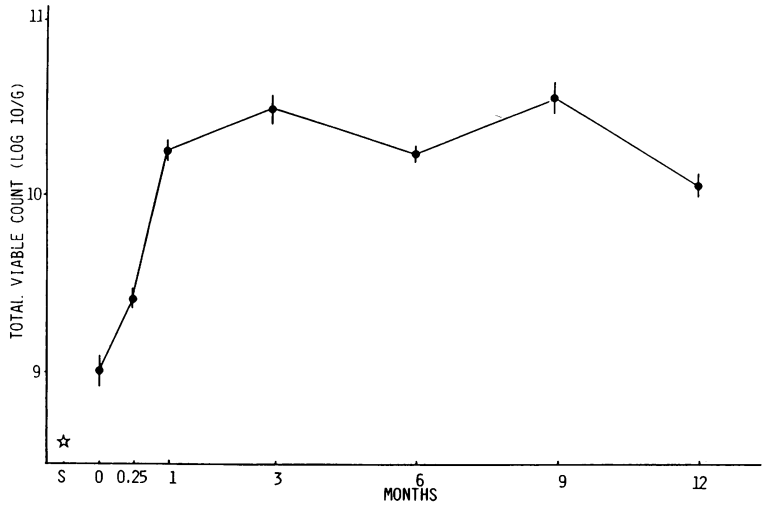

FIG. 1. Total viable count of microorganisms per gram of plaque from SUS rats (mean ± standard deviation). S, Viable count of organisms in saliva.

giva) was 1, mild inflammation with extensive swelling and change to pale pink or dark red; 2, moderate inflammation with extensive swelling, change to dark red, and pocket formation; and 3, severe inflammation with extensive pronounced swelling, change to dark red, and clear pocket formation.

Pathological examinations. Inflamed gingival tissue in the lower incisor area of SUS rats and the same portion in the gingiva of RES rats was obtained from the lower incisor area. Specimens were fixed in Formalin, demineralized in 7.5% HNO_3 solution, embedded in paraffin, and processed for light microscopy.

RESULTS

Effect on contact infection and plaque inoculation on RES rats. The infection could not be transmitted to the RES rats by either cohabitation with SUS rats or direct inoculation of SUS plaque onto the gingiva of RES rats.

Pregingivitis flora of SUS and RES rats. Pregingivitis flora of SUS and RES rats was examined (Table 1). The predominant types of bacteria were *Streptococcus* spp., *Lactobacillus* spp., *Veillonella* spp., and *Neisseria* spp. In particular, *Streptococcus* spp. constituted the major flora in both strains of rats. *Streptococcus salivarius* was found in high proportions on the saliva, tongue dorsum, and buccal mucosa. *Streptococcus mitis* was found in high proportions on the tongue dorsum and buccal mucosa. *Streptococcus sanguis* was also found in the oral cavity. On the other hand, *S. mutans* was not found on the saliva, tongue dorsum, and buccal mucosa and was found in low proportions on the gingival crevice. Gram-negative bacteria, such as *Bacteroides* spp., were found in low proportions. The oral flora of SUS rats was similar to that of RES rats.

Counts of microbial groups in plaque samples of SUS rats. The total viable counts of bacteria in plaque samples from SUS rats increased from ca. 10^9 to 10^{11} cells per g (wet weight) after the rats were fed a commercial powder diet (Fig. 1). The counts of bacteria in the plaque samples were always higher than those of bacteria in the saliva. In SUS rats, the increase in the total counts of bacteria corresponded to an increase in plaque volume.

Viable counts in anaerobic EG agar, TF agar, and GAM

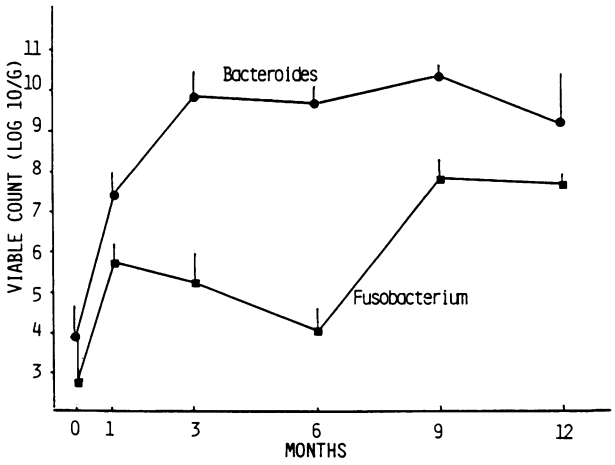

FIG. 2. Number of *Bacteroides* and *Fusobacterium* species per gram of plaque from SUS rats (mean ± standard deviation).

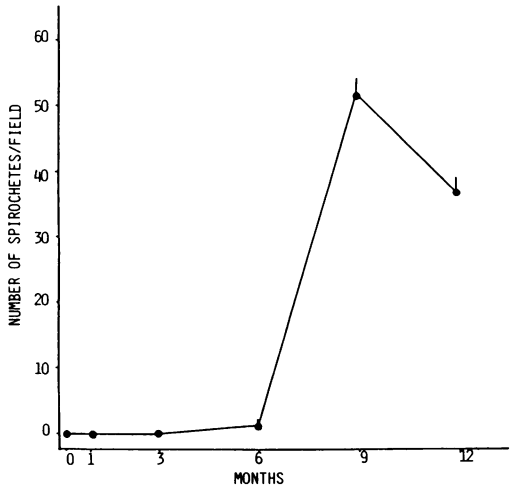

FIG. 3. Number of *Spirochetes* species in plaque solution (1 mg/ml) (mean ± standard deviation).

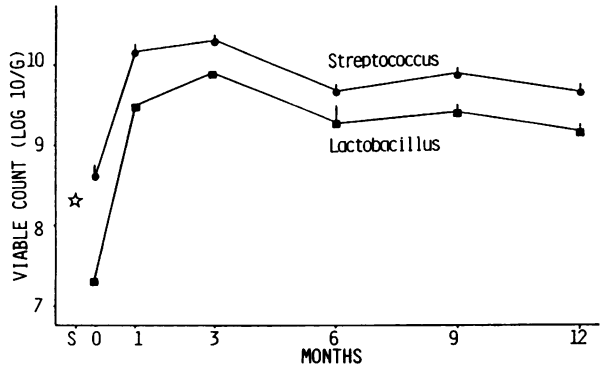

FIG. 4. Number of *Streptococcus* and *Lactobacillus* species per gram of plaque from SUS rats (mean ± standard deviation).

agar plates were similar to each other and lower than the direct count, constituting 39 to 75% (mean, 64%) of the direct count.

The number of *Bacteroides* species significantly increased and became a predominant population in the first 3 months (Fig. 2). *Fusobacterium* species and oral *Treponema* species were recognized in increasing numbers, especially at 9 months (Fig. 2 and 3). The count of *Streptococcus* and *Lactobacillus* species increased in the plaque from 0 to 1 month and then remained constant between 2 and 12 months (Fig. 4). The counts of *Veillonella* species increased similarly to those of *Lactobacillus* species. The proportions of cultivable flora in plaque changed between 0 and 12 months after the rats were fed a powder diet (Table 2). Pregingivitis flora of the gingival crevice consisted of *Streptococcus* spp., *Lactobacillus* spp., *Veillonella* spp., *Neisseria* spp., and other species. With development of plaque, certain of the different populations, such as *Streptococcus* and *Neisseria* species, decreased, whereas *Bacteroides* species increased in relative proportions. *Fusobacterium* species were present in greater proportions at 6 and 9 months than they were in pregingivitis flora. Although members of the family *Enterobacteriaceae* were sometimes isolated from SUS rats, there appeared to be no relationship with plaque age.

TABLE 2. Changes in the proportions of cultivable flora in plaque from SUS rats

Bacteria in plaque from SUS rates	% Pregingivitis flora	% Cultivable flora in plaque at month:					
		0	1	3	6	9	12
Gram-negative rods							
Bacteroides spp.	1.4	2.0	5.5	14.4	26.7	30.8	24.6
B. intermedius	0	0	<1	1.9	NT[a]	7.2	NT
B. melaninogenicus	0	0	<1	0.3	NT	0.3	NT
B. gingivalis	0	0	0	0	NT	0	NT
Fusobacterium spp.	0.2	0.6	0.3	0.3	1.3	2.5	0.5
Enterobacteriaceae	5.6	0	0	7.3	1.0	2.8	0.8
Others	10.2	3.1	7.9	8.0	8.1	10.1	6.6
Gram-positive cocci							
Streptococcus spp.	59.9	62.1	62.0	45.0	27.0	21.7	34.2
Staphylococcus spp.	0	0	0	<0.1	0	0.9	0
Others	0.1	0.1	0.2	0.2	<0.1	<0.1	<0.1
Gram-positive rods							
Lactobacillus spp.	9.1	17.5	10.9	5.2	15.1	11.9	14.4
Others	0.2	0.8	1.9	0.6	2.2	3.7	1.7
Gram-negative cocci							
Veillonella spp.	7.3	12.1	10.3	18.8	18.4	15.4	17.0
Neisseria spp.	5.8	1.8	1.0	0.2	1.4	0.2	0.3
Others	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1

[a] NT, Not tested.

Identification of isolated bacteria. Isolates were classified into groups by the scheme presented in Table 1. Gram-negative rods were frequently isolated. Black-pigmented *Bacteroides* spp., such as *B. intermedius* and *B. melaninogenicus*, were identified by their biochemical characteristics. The antigenicity of some isolated *Bacteroides* spp. could be confirmed with immunodiffusion by comparison with standard antigens and antisera prepared from known *Bacteroides* strains (Fig. 5).

Some isolated strains reacted strongly, forming at least two precipitin bands, with the antiserum against *B. intermedius* or *B. melaninogenicus* in immunodiffusion. Some isolated strains formed a faint precipitin band, which was considered to be a cross-reaction between species. SDS-extracted antigens from isolated *Bacteroides* species did not react with anti-B-LPS antiserum. Other *Bacteroides* species (ca. 80%) could not be identified by biochemical tests and immunodiffusion. *Fusobacterium nucleatum*, *Haemophilus* species, and *Streptobacillus* species were also detected.

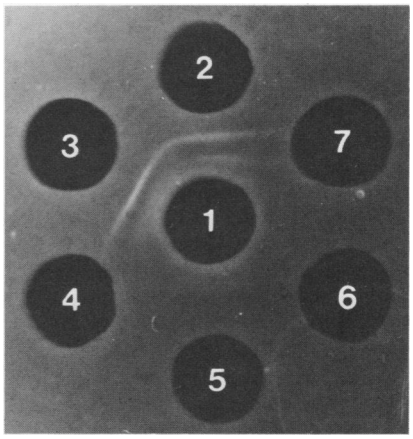

FIG. 5. Immunodiffusion test in gel. The immune serume is in well 1 (anti-*B. melaninogenicus* antiserum). The antigens are in well 2 (SDS-extracted antigen from *B. melaninogenicus*) and wells 3 through 7 (SDS-extracted antigens from isolated *Bacteriodes* spp.). Antigen in well 3 reacted strongly against the anti-*B. melaninogenicus* in well 1. Antigen in well 5 formed a faint precipitin band against anti-*B. melaninogenicus*. Antigens in wells 4, 6, and 7 formed no precipitin band. Anti-*B. melaninogenicus* antiserum reacted strongly, forming at least two precipitin bands, with SDS-extracted antigen of homologous strains, whereas the antiserum did not react with the other standard antigens, with the exception of formation of a faint band. Similarly, standard antiserum against *B. intermedius*, *B. levii*, *B. gingivalis*, *B. oralis*, and *B. loescheii* also showed a strong reaction with homologous strains but not with heterologous strains.

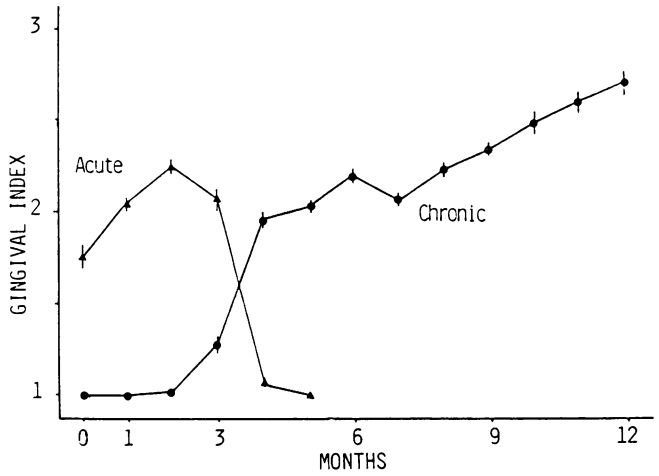

FIG. 6. Changes of the gingival index in SUS rats (mean ± standard deviation). For definition of the criteria for the gingival index system, see the text.

Members of the family *Enterobacteriaceae* were also seen in the plaque. Gram-negative cocci, such as *Veillonella* and *Neisseria* spp., were sometimes isolated. Gram-positive rods, such as *Corynebacterium* and *Propionibacterium* spp., and gram-positive cocci, such as *S. salivarius*, were also seen in the plaque. By Gram staining of the smear of the plaque, it was seen that gram-positive cocci and rods were present in the surface of the plaque and not in anaerobic subgingival pockets.

Gingivitis in SUS rats. The incidence of acute inflammation as determined by the gingival index increased between 0 and 2 months (Fig. 6). The inflammation of the gingiva progressed from acute to chronic. The incidence of chronic gingivitis as indicated by the gingival index increased between 2 and 12 months.

Histopathologically, acute gingivitis was observed within 3 months and subacute-chronic gingivitis was observed between 2 and 12 months in SUS rats (Fig. 7). The former was characterized by polymorphonuclear leukocyte infiltration and edema, and the latter was characterized by plasma cell infiltration.

In RES rats, the gingival index showed a value of 0 between 0 and 12 months. No inflammation of the gingiva was observed histopathologically.

Plaque formation. Plaque formation was recognized in SUS rats but not in RES rats (Fig. 8). The plaque volume increased gradually with time in SUS rats.

DISCUSSION

The present studies examined the development of gingivitis in relation to various oral bacteria. The development of gingivitis was paralleled by an increase in the volume of gingival plaque and the predominance of anaerobic microflora. Gram-negative anaerobic bacteria, such as *Bacteroides* spp., were isolated in increased numbers. Similar

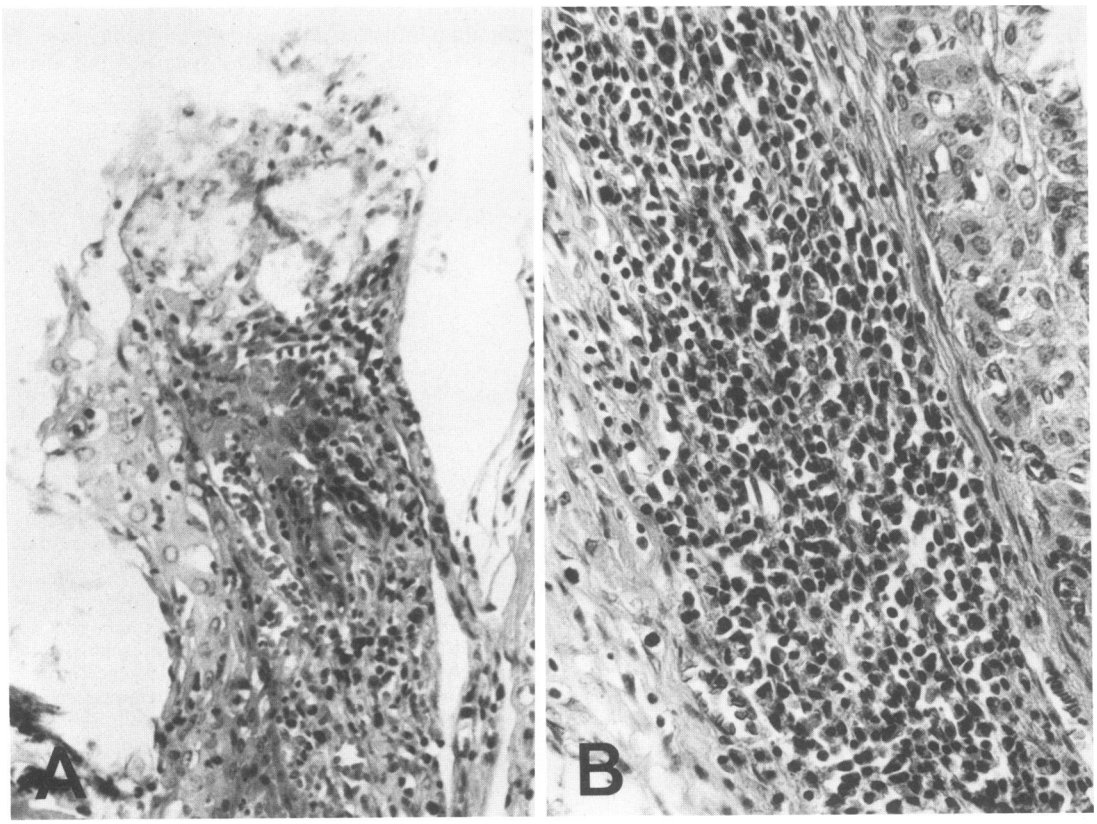

FIG. 7. Inflammation of the gingiva. (A) Acute gingivitis was observed in the gingiva of 3-month-old SUS rats. A large number of polymorphonuclear cells infiltrated into the gingival tissue with destruction of epitherial cells. Magnification, ×230. (B) Subacute-chronic gingivitis was observed in the gingiva of 9-month-old SUS rats. A large number of plasma cells infiltrated into the gingival tissue. Magnification, ×600.

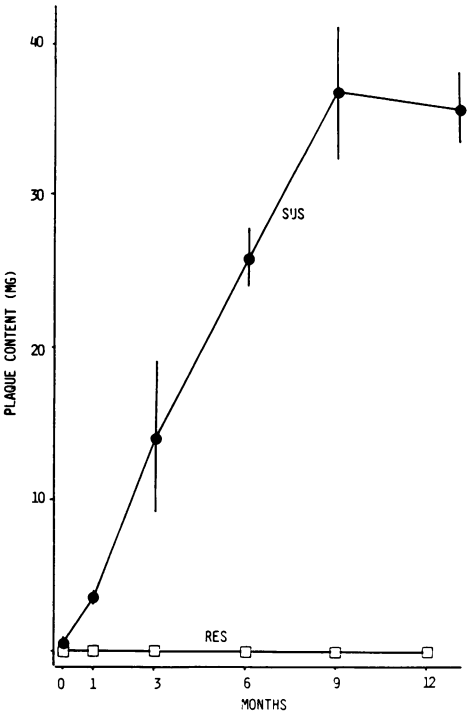

FIG. 8. Changes in the plaque content for each experimental period in SUS rats (mean ± standard deviation).

findings have been obtained with human gingivitis and periodontitis (15, 22).

In SUS rats, a close relationship was found between the accumulation of bacterial plaque and gingivitis. In the process of plaque accumulation, the bacterial flora changed from a predominantly gram-positive coccal form to a complex population of spirochetes and gram-negative bacteria. Interactions between plaque score, gingivitis score, and flora have been demonstrated (15). These findings raise the possibility that proportional changes in the gingival plaque flora and plaque volume contribute to the development of gingival inflammation.

In the present study, *B. intermedius* and *B. melaninogenicus* were isolated from the plaque of SUS rats showing severe gingivitis. In previous studies, these bacteria were shown to produce LPS and other toxic products capable of destroying tissue and were pathogenic in experimental infections (5, 21, 23). It has been suggested that black-pigmented *Bacteroides* spp., such as *B. intermedius*, are important in the development of gingivitis. Similar *Bacteroides* species were isolated in high numbers from humans with periodontal diseases (19, 30).

Streptococci constituted ca. 62.1% of the plaque flora at month 0, but as the plaque aged to 9 months, the proportional contribution of the streptococci of the total plaque flora decreased to 21.7%. Streptococci near the plaque surface may have been the initial colonizers of the plaque and then provided the anaerobic conditions in this model.

The results of this investigation indicate that the proportion of *Neisseria* spp., which was high initially, declined with the progression of plaque development. The anaerobic bacteria, such as *Bacteroides* spp., significantly increased in proportion (1.4% in pregingivitis flora compared with 30.8% in plaque that had aged 9 months).

In this study, *Spirochetes* and *Lactobacillus* species and enterococci were isolated from SUS rat plaque, and changes in the bacterial flora were detected. Azuma et al. reported that no *Spirochetes* and *Lactobacillus* species or enterococci could be isolated from SUS rat plaque (2), and no change in the bacterial flora during plaque formation could be detected (1); moreover, the other bacteria involved in SUS rat plaque were different. The following reasons may explain these apparent discrepancies. First, we examined the SUS plaque

TABLE 3. Identification of bacteria isolated from the plaque of SUS rats

Group	Test results
Gram-negative rods	
B. intermedius	Only anaerobic growth, black pigment, motility −, esculin −, indole +, gelatin −, glucose +, lactose −, rhamnose −, trehalose −, cellobiose −, sorbitol −, arabinose −, maltose +, salicin −, xylose −, mannose −, raffinose −, glycerol −, mannitol −, sucrose −, catalase −, nitrate −, urease −, bile −
B. melaninogenicus	Only anaerobic growth, black pigment, motility −, esculin +, indole −, gelatin −, glucose +, lactose +, rhamnose −, trehalose −, cellobiose −, sorbitol −, arabinose −, maltose −, salicin +, xylose −, mannose −, raffinose −, glycerol −, mannitol −, sucrose −, catalase −, nitrate −, urease −, bile −
Undefined *Bacteroides* spp.	
F. nucleatum	Only anaerobic growth, growth on *Fusobacterium* selected agar, spindle-shaped, tapered ends, indole +, glucose +, other tests were negative
Others	
Haemophilus influenza	
Streptobacillus moniformis	
Escherichia coli	
Citrobacter freundii	
Gram-positive cocci	
S. mutans	MS +, MSB +, mannitol +, glucose +, sorbitol −, arginine −, esculin +, catalase −, VP +
S. sanguis	MS +, MSB −, mannitol −, glucose +, sorbitol −, arginine +, esculin +, catalase +, VP −
S. mitis	MS +, MSB −, mannitol −, glucose −, sorbitol −, arginine −, esculin −, catalase +, VP +
S. salivarius	MS +, MSB −, mannitol −, glucose −, sorbitol −, arginine −, esculin +, catalase −, VP +
Staphylococcus spp.	
Gram-positive rods	
Lactobacillus acidophilus	
Corynebacterium spp.	
Propionibacterium spp.	
Gram-negative cocci	
Veillonella spp.	
Neisseria spp.	

for 12 months and tried to identify the isolated bacteria, whereas Azuma et al. examined the plaque for only 3 months and did not try to identify the bacteria. Second, the rats used in the present study have been inbred for over 20 generations, and this may have affected their susceptibility to the accumulation of plaque.

The percentage of *Enterococcus* spp. appeared to have no relationship to plaque age. The finding of *Enterococcus* spp. indicated that the fecal flora of the rats contaminated the plaque as a result of coprophagy. *Corynebacterium* and *Propionibacterium* spp. were minor flora of the oral cavity of the rats. There was no relationship between the proportions of flora and the progression of gingivitis.

The *Lactobacillus* counts are high in this study. This is in sharp contrast to human studies, in which lactobacilli are rarely isolated from periodontal plaques (6). In humans *Actinomyces* and *Eubacterium* spp. are frequently encountered (3, 17, 26). Differences in the oral environment, such as pH, may induce different oral microbial flora.

The bacterial counts in plaque are from 10^9 to 10^{11} per g. As plaque is considered to be entirely composed of bacteria, with a density of ca. 10^{11} cells per g (wet weight), it is difficult to envision a plaque that is only 1% bacteria, as would be the case with 10^9 cells per g of plaque. This could be explained in two ways: first, the small amount of plaque (ca. 1 mg) was collected with contamination by saliva, and second, the plaque at 0 time was composed of the powder diet with a small percentage of bacteria.

It has been noticed that SUS rats can be divided into at least three groups, the severe recurrent acute gingivitis group, the acute-subacute-chronic gingivitis group, and the long-term mild gingivitis group. However, it was difficult to divide the rats into three clear groups, and intermediates appeared. The *Fusobacterium* viable count was ca. 10^4/g at 6 months and increased to 10^8/g at 9 months (Fig. 2). This is shown in Table 2 as an increase from 1.3% of the cultivable count at 6 months to 2.5% at 9 months. It was considered that these different data were due to the use of different groups of rats.

The identification of anaerobic bacteria is often based on the criteria given in the *Anaerobic Laboratory Manual* by Holdeman et al. from the Virginia Polytechnic Institute (7). In this manual, taxonomic criteria are based on morphological appearance, fermentation pattern, and analysis of fatty acids and products of glucose metabolism by gas-liquid chromatography. In the present study, the fermentation pattern was examined by the Minitek system, and immunodiffusion was used as a supplementary test to determine the species of anaerobic bacteria such as *Bacteroides* spp. The Minitek system does not have rat isolates in the data base, and the identifications are estimations based on phenotypically similar bacterial species. Immunodiffusion was sufficient to identify some *Bacteroides* species. Nevertheless, other *Bacteroides* species remained undefined. Further studies on the species-specific antigen of *Bacteroides* spp. will be necessary.

Periodontal disease is a multifactorial disease. Dental plaque, however, has been found to be the most important causative factor. The present results suggest that *Bacteroides* spp. may have an important role in the destruction of gingival tissue. *Spirochetes* and *Fusobacterium* species and other bacteria could be additional exacerbating factors in the rat model.

The existence of SUS and RES rats implies that some host factors have resulted in the animals being colonized by gram-negative anaerobic bacteria. RES rats showed no plaque accumulation and gingivitis after contact infection or plaque inoculation. Furthermore, proportions of predominant oral bacteria of SUS rats were similar to those of RES rats at 3 weeks of age. Studies of host factors are now in progress.

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LITERATURE CITED

1. **Azuma, Y., N. Ito, M. Shinohara, and M. Mori.** 1978. Experimental gingivitis in ODU plaque-susceptible rats. II. Biochemical nature of the rat plaque. J. Periodontol. **49**:60–63.
2. **Azuma, Y., M. Shinohara, N. Ito, and M. Mori.** 1979. Experimental gingivitis in ODU plaque-susceptible rats. J. Periodontol. **50**:416–418.
3. **Darwish, S., T. Hyppa, and S. S. Socransky.** 1978. Studies of the predominant cultivable microbiota of early periodontitis. **13**:1–16.
4. **Genco, R. J., and J. Slots.** 1984. Host responses in periodontal diseases. J. Dent. Res. **63**:441–451.
5. **Gibbons, R. J., and J. B. Macdonald.** 1961. Degradation of collagenase substrates by *Bacteroides melaninogenicus*. J. Bacteriol. **81**:614–621.
6. **Hardie, J.** 1983. Microbial flora of the oral cavity, p. 162–196. *In* Oral microbiology and infectious disease.
7. **Holdeman, L. V., E. P. Cato, and W. E. C. Moore.** 1977. Anaerobic laboratory manual, 4th ed. Virginia Polytechnic Institute and State University, Blacksburg.
8. **Ito, N., Y. Azuma, and M. Mori.** 1975. Experimental gingivitis. Development of a new strain of plaque-susceptible rats. J. Dent. Res. **54**:425.
9. **Ito, N., M. Shinohara, and M. Mori.** 1977. Experimental gingivitis in ODU plaque-susceptible rats. I. Changes of plaque formation and body weight. J. Periodontol. **48**:201–208.
10. **Kitamura, M., H. Kaneko, N. Sasaki, and N. Ito.** 1982. Changes of sucrose and glucose contents in adherent plaque of plaque-susceptible rat. Higashi Nippon Gakuen Univ. J. **1**:45–51. (In Japanese.)
11. **Lindhe, J., and S. Nyman.** 1975. The effect of plaque control and surgical pocket elimination on the establishment and maintenance of periodontal health. A longitudinal study of periodontal therapy in case of advanced periodontis. J. Clin. Periodontol. **2**:67–74.
12. **Listgarten, M. A., and L. Hellden.** 1978. Relative distribution of bacteria at clinically healthy and periodontally diseased sites in humans. J. Clin. Periodontol. **5**:115–132.
13. **Löe, H.** 1967. The gingival index, the plaque index and the relation index systems. J. Periodontol. **38**:610–616.
14. **Löe, H., E. Theilade, and S. B. Jensen.** 1965. Experimental gingivitis in man. J. Periodontol. **36**:177–187.
15. **Loesche, W. J., and S. A. Syed.** 1978. Bacteriology of human experimental gingivitis: effect of plaque and gingivitis score. Infect. Immun. **21**:830–839.
16. **Mitsuoka, T., T. Sega, and S. Yamamoto.** 1965. Eine verbesserte Methodik der qualitativen und quantitativen Analyse der Darmflora von Menschen und Tieren. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **195**:455–469.
17. **Moore, W. E. C., R. R. Ranney, and L. V. Holdeman.** 1982. Subgingival microflora in periodontal disease: cultural studies, p. 13–26. *In* R. J. Genco and S. E. Mergenhagen (ed.), Host-parasite interactions in periodontal disease. American Society for Microbiology, Washington, D.C.
18. **Parker, C. A.** 1955. Anaerobiosis with iron wool. Aust. J. Exp. Biol. Med. Sci. **33**:33–38.
19. **Slots, J.** 1977. The predominant cultivable microflora of advanced periodontitis. Scand. J. Dent. Res. **85**:114–121.
20. **Slots, J.** 1979. Subgingival microflora and periodontal disease. J. Clin. Periodontol. **6**:351–382.
21. **Slots, J.** 1982. Importance of black-pigmented *Bacteroides* in

human periodontal disease, p. 27–45. *In* R. J. Genco and S. E. Mergenhagen (ed.), Host-parasite interactions in periodontal disease. American Society for Microbiology, Washington, D.C.

22. **Slots, J., and R. J. Genco.** 1984. Black-pigmented *Bacteroides* species, *Capnocytophaga* species and *Actinobacillus actinomycetemcomitans* in human periodontal disease: virulence factors in colonization, survival and tissue destruction. J. Dent. Res. **63**:412–421.
23. **Socransky, S. S.** 1977. Microbiology of periodontal disease—present status and future considerations. J. Periodontol. **48**:497–504.
24. **Stargel, M. D., F. S. Thomson, S. E. Phillips, G. L. Lombard, and V. R. Dowell, Jr.** 1976. Modification of the Minitek miniaturized differentiation system for characterization of anaerobic bacteria. J. Clin. Microbiol. **3**:291–301.
25. **Suomi, J. D., and J. Doyle.** 1972. Oral hygiene and periodontal disease in adult population in the United States. J. Periodontol. **43**:677–681.
26. **Syed, S. A., and W. J. Loeshe.** 1978. Bacteriology of human experimental gingivitis: effect of plaque age. Infect. Immun. **21**:821–829.
27. **Westphal, O., O. Luderitz, and F. Bister.** 1952. Über die Extraktion von Bakterien mit Phenol/Wasser. Z. Naturforsch. **76**:148–155.
28. **White, D., and D. Mayrand.** 1981. Association of oral Bacteroides with gingivitis and adult periodontitis. J. Periodontal Res. **16**:259–265.
29. **Wilcox, W. R., and S. P. Lapage.** 1975. Methods used in a program for computer-aided identification of bacteria, p. 103–119. *In* R. J. Pankhurst (ed.), Biological identification with computers. Academic Press, Inc., New York.
30. **Zambon, J. J., H. S. Reynolds, and J. Slots.** 1981. Black-pigmented *Bacteroides* spp. in the human oral cavity. Infect. Immun. **32**:198–203.