The Effect of Intensive Antibacterial Therapy on the Sulcular Environment in Monkeys*

Part I. Changes in the Bacteriology of the Gingival Sulcus

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THE CHANGES INDUCED in the bacteriology of the gingival sulcus were evaluated as part of a study considering the keratinizing potential of the sulcular epithelium when bacterial plaque was essentially eliminated.

Two Rhesus monkeys were scaled and placed on a daily therapeutic regimen which included a prophylaxis, systemic tetracycline, and topical chlorhexidine. Over the 40 day experimental period and 74 days post-therapy, subgingival plaque samples were taken periodically. The plaque samples were cultured anaerobically and aerobically to determine the predominant bacterial flora.

The total cultivable bacterial flora decreased from initial levels by greater than 99.9% with the antibacterial therapy. The flora shifted with therapy from one dominated by anaerobic organisms, including *Bacteroides melaninogenicus* (18%) and *Fusobacterium* species (13.9%), to a flora dominated by organisms growing aerobically. During treatment *B. melaninogenicus* and *Fusobacterium* species were not detected in any sample. After cessation of all therapy the anaerobes increased to dominance again, but *B. melaninogenicus* remained undetectable through 74 days post-therapy.

Replacement of the keratinized stratified squamous epithelium is a continual process involving cells in four discrete stages of development, based on morphological criteria, arranged in layers parallel to the surface. Cells appear to divide, differentiate and migrate into a final surface layer which is generally thought to be a protective barrier.

Since periodontal disease appears to begin as a bacterial plaque-induced inflammation subjacent to the sulcular and junctional epithelium,[1,2] the lack of a keratin layer in these areas has been interpreted as an inherent weakness in the human gingival sulcus. Therefore, many studies have attempted to define the nature of the sulcular epithelium.[3-9] The keratinizing potential for this tissue has been demonstrated recently in surgical studies which removed the sulcular epithelium from approximating the tooth.[3,4] It seems appropriate at this time to evaluate the keratinizing potential of sulcular epithelium which remains *in situ* but for which the sulcular environment is greatly altered.

This report is part of a study evaluating the keratinizing potential of sulcular epithelium when bacterial plaque is essentially eliminated. The changes induced in the bacteriology of the gingival sulcus will be presented here.

MATERIALS AND METHODS

Two adult male Rhesus monkeys (macaca mulatta), each weighing approximately 17 pounds, were selected for the present study. Both had a full complement of permanent teeth with moderate accumulations of supragingival and subgingival calculus and with moderate generalized gingivitis.

Sodium pentobarbital, 30 mg/kg body weight IV, was used for all surgical procedures. The monkeys were sedated with ketamine hydrochloride, 200 mg IM, for the daily prophylaxes.

Three months prior to the study, two marginal plaque samples were removed from each monkey and bacterial isolates were tested for antibiotic sensitivity. Clinical procedures were performed as outlined in Table 1. At the initiation of the study (Day = 0) two plaque samples were taken from each monkey and gingival biopsies were taken at the sample sites. This was followed by scaling and polishing to remove all subgingival calculus and plaque.

Antibacterial therapy was initiated on the day of scal-

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ing and included the following procedures once daily for 40 days:

1. Tetracycline hydrochloride (Achromycin®) 75 mg IV.
2. Rubber cup prophylaxis with prophylaxis paste (Nupro®). Not performed on weekends.
3. Chlorhexidine gluconate 0.2% in a nonionic gel applied to the sulcus area by means of syringe and irrigation needle. The chlorhexidine was not washed off after application. Not performed on weekends.

Additional plaque samples and biopsies were taken from the buccal aspect of different teeth in both monkeys at the times indicated in Table 1.

Bacteriology

Plaque samples were removed with a sterile Columbia 13-14 curette.|| The curette was inserted to the base of the sulcus. The tooth surface was engaged with the blade and the curette was moved to the orifice of the sulcus with a single sweeping motion. The removed plaque was placed in a 1 dram glass vial containing 1.5 ml of reduced transport fluid (RTF).[10] The prereduced RTF was added to the vials within an anaerobic glove box,[11] and the vials were sealed and the caps taped prior to removal for sampling. This was intended to maintain the RTF in a reduced state until the sample was taken.

The plaque samples were transported to the Oral Bacteriology Laboratory and placed in the anaerobic chamber within 1 hour of sampling. All of the following manipulations were performed within the anaerobic chamber. Samples were dispersed for 20 seconds by means of a Kontes Sonifier Model K-881440 set for maximum output.

Sterile disposable tuberculin syringes were used to remove 0.5 ml of the dispersed sample and to make serial 1/20, 1/100, and 1/1000 dilutions in RTF. The dilutions were plated on selective and nonselective media by means of an automatic diluting and plating device.¶

Media

Dilutions were plated on the following selective and nonselective media:

ETSA: Enriched trypticase soy agar. This is a nonselective blood agar enriched with menadione (1 μg/ml) and hemin (2 μg/ml).

GMC: Gelatin-metronidazole (10 μg/ml) Cadmium sulfate (20 μg/ml) agar. This selective medium allows isolation and presumptive identification of *Actinomyces viscosus* and *Actinomyces naeslundii.*[12]

ETSA-K: ETSA with 50 μg/ml Kanamycin sulfate (Kantrex[t]) for selection of gram-negative organisms.

MM10 with 5% sucrose: The M10 rumen fluid formulation of Caldwell and Bryant (1966) modified to include known nutrient requirements of the oral flora and to allow presumptive identification of *Streptococcus sanguis* and *Streptococcus mutans.*[10]

All media were prereduced in the chamber for at least 24 hours immediately prior to use. Dilutions were plated as follows:

1/20: ETSA (2), ETSA-K, GMC, MM10
1/100: ETSA (2), ETSA-K, GMC, MM10
1/1000: ETSA (2)

One ETSA plate from each dilution was incubated at 37°C in an aerobic incubator. All other plates were incubated in the anaerobic chamber,* and maintained as described by Aranki et al.[11] with the oxygen level held at less than 10 parts per million as determined biweekly by means of a trace oxygen analyzer.†

Anaerobic plates were incubated for 7 days. For each sample, dilutions which gave plates with well dispersed colonies were selected.

Sectors including more than 20 colonies were selected and colonies in two such sectors were quantitated by means of a binocular dissection microscope. The total number of colonies and the number of selected colony types were determined for each sector. Differential counts and biochemical characterizations of isolates were keyed to those organisms currently thought to undergo proportional shifts associated with periodontal disease.

These organisms are:
Actinomyces naeslundii
Actinomyces odontolyticus
Actinomyces viscosus
Bacteroides melaninogenicus (three subspecies)
Bacteroides (Capnocytophaga) ochraceus

Table 1.
Experimental Protocol

Time	Plaque samples	Biopsies	Additional procedures
days	*Number*	*Number*	
0*	4	4	
3	4	4	
5	4	4	
7	4	4	
10	4	4	
13	4	4	
19	4	4	
27	4	4	
33	4	2	2 Block sections
40	4	2	2 Block sections
Post-therapy			
7	4	4	
23	4	4	
36	2	2	
74	2	2	

* Day 0 samples taken immediately prior to initiation of scaling and antibacterial therapy.

|| Star Manufacturing Company, Philadelphia, PA.
¶ Spiral Systems, Cincinnati, OH.

* Supplied by Coy Manufacturing Company, Ann Arbor, MI.
† Lockwood & McLorie, Inc.

Fusobacterium species
Streptococcus sanguis
Surface translocating bacteria (STB)

Isolates were classified according to the following criteria:

1. Colony morphology
2. Gram-stain morphology
3. Aerobic growth
4. Indole production
5. Esculin hydrolysis
6. Gelatinase activity
7. Catalase activity
8. pH in glucose broth
9. pH in glucose-1-phosphate broth
10. pH in mannitol broth
11. Acid end products by gas liquid chromatography
12. Motility by dark field microscopy
13. Nitrate reduction
14. Nitrite reduction

RESULTS

Bacteriology

Prior to treatment moderate accumulations of supragingival and subgingival plaque were evident in all sites. This pretreatment plaque bacterial flora of the monkey was found to be highly sensitive to tetracycline, as evaluated by the modified disc diffusion method.[13]

The initial plaque samples averaged more than 10^8 cultivable organisms per sample. These samples were dominated by bacteria which preferred anaerobic growth conditions, as evidenced by the anaerobe/aerobe ratio of 3.20. Differential counts of the cultivable flora revealed *Bacteroides melaninogenicus* (18.2%), *Fusobacterium* species (13.9%) and *Streptococcus sanguis* (22.4%) as major components (Table 2).

The *B. melaninogenicus* isolates lowered the pH below 6.0 in glucose broth, produced indole from tryptophane, and failed to hydrolyze esculin. They were therefore classified as subspecies *intermedius.* A few isolates at 0 time were compatible with *B. melaninogenicus* subspecies *asaccharolyticus* (*Bacteroides asaccharolyticus*).

Antibacterial therapy included daily tetracycline IV, and prophylaxis and chlorhexidine applications daily on weekdays. This therapy produced an absolute decrease in the cultivable flora from 10^8 organisms/sample prior to treatment to 10^4 organisms/sample after 40 days of treatment. These data indicate that more than 99.9% of the plaque flora was eliminated.

The anaerobe/aerobe ratio decreased markedly after initial preparation of the animals but increased again to approximately 1.90 by 7 days. By Day 19 the anaerobe/aerobe ratio had started to decline and remained below 1.00 from Day 27 to the termination of the antibacterial therapy 13 days later (Table 2, Fig. 1).

Actinomyces viscosus, Actinomyces naeslundii, Bacteroides melaninogenicus, and *Bacteroides* (*Capnocytophaga*) *ochraceus* could not be detected in samples 3 days after therapy began and remained undetectable throughout the treatment. Absolute numbers of *Streptococcus sanguis* declined during treatment, but this organism became dominant in samples taken early in the treatment. In later samples, *S. sanguis* shared prominence with another streptococcus which was adherent and refractile on 5% sucrose agar, did not lower the pH in mannitol broth, and did not hydrolyze esculin. This organism is compatible with *S. sanguis* II as described by Facklam.[14]

Bacterial counts, after cessation of all treatment, increased significantly from 10^4 cultivable organisms/ml sample to 9×10^6 organisms/ml sample 36 days after ending treatment. The anaerobe/aerobe ratio increased to approximately 2.0 within the first week after stopping

Table 2.
Plaque Bacterial Flora Changes With the Progress of Antibacterial Therapy

Time*	N†	Total CFU‡ (× 10^5)	Anaerobe/ Aerobe§	*Actinomyces viscosus*	*Actinomyces naeslundii*	*Bacteroides melaninogenicus*‖	*Fusobacterium* species	*Bacteroides ochraceus*	*Streptococcus sanguis*
days									
0	4	1045 ± 268	3.20 ± 0.46	0.40 ± 0.14¶	0.20 ± 0.08	18.2 ± 1.97	13.9 ± 1.58	6.67 ± 0.93	22.4 ± 4.20
3	4	5.39 ± 2.20	0.65 ± 0.11	ND**	ND	ND	ND	ND	66.8 ± 3.45
5	4	0.69 ± 0.29	0.87 ± 0.11	ND	ND	ND	ND	ND	60.3 ± 4.80
7	4	0.70 ± 0.15	1.92 ± 0.35	ND	ND	ND	ND	ND	72.5 ± 5.02
13	4	1.07 ± 0.44	1.86 ± 0.09	ND	ND	ND	ND	ND	68.3 ± 3.48
19	4	0.56 ± 0.18	1.32 ± 0.34	ND	ND	ND	ND	ND	87.3 ± 6.42
27	4	0.28 ± 0.05	0.76 ± 0.22	ND	ND	ND	ND	ND	38.5 ± 8.44
33	4	0.54 ± 0.37	0.73 ± 0.16	ND	ND	ND	ND	ND	31.6 ± 4.80
40	4	0.10 ± 0.05	0.79 ± 0.07	ND	ND	ND	ND	ND	23.5 ± 4.23

* Time 0 samples taken prior to initiation of scaling and antibacterial therapy.
† Number of plaque samples cultured.
‡ Total colony forming units (CFU) on a nonselective medium incubated anaerobically, ± standard deviation.
§ Ratio of total CFU of duplicate nonselective plates, one incubated anaerobically and the other aerobically.
‖ Includes three subspecies.
¶ Percent of total CFU, ± standard deviation.
** Not detectable; less than 1035 organisms in the total plaque sample.

treatment. By 36 days post-treatment, the ratio had exceeded pretreatment levels and continued to increase through 74 days post-treatment (Table 3, Fig. 1).

Actinomyces viscosus remained undetectable in post-treatment samples, but *Actinomyces naeslundii* increased to approximately 18% of the cultivable flora. Only two plaque samples were cultured at the last period, and the mean is not truly representative of the levels (34.0% and 2.7%) of *A. naeslundii* in the individual samples. *B. melaninogenicus* was undetectable in all post-treatment samples. *Fusobacterium* species increased from undetectable to approximately 40% of the flora and *B. (Capnocytophaga) ochraceus* increased to approximately pretreatment levels (Table 3).

DISCUSSION

The plaque bacterial flora of the Rhesus monkey appears qualitatively similar to that described for man. The flora associated with chronic gingivitis in man generally has higher levels of *A. viscosus* and *A. naeslundii*,

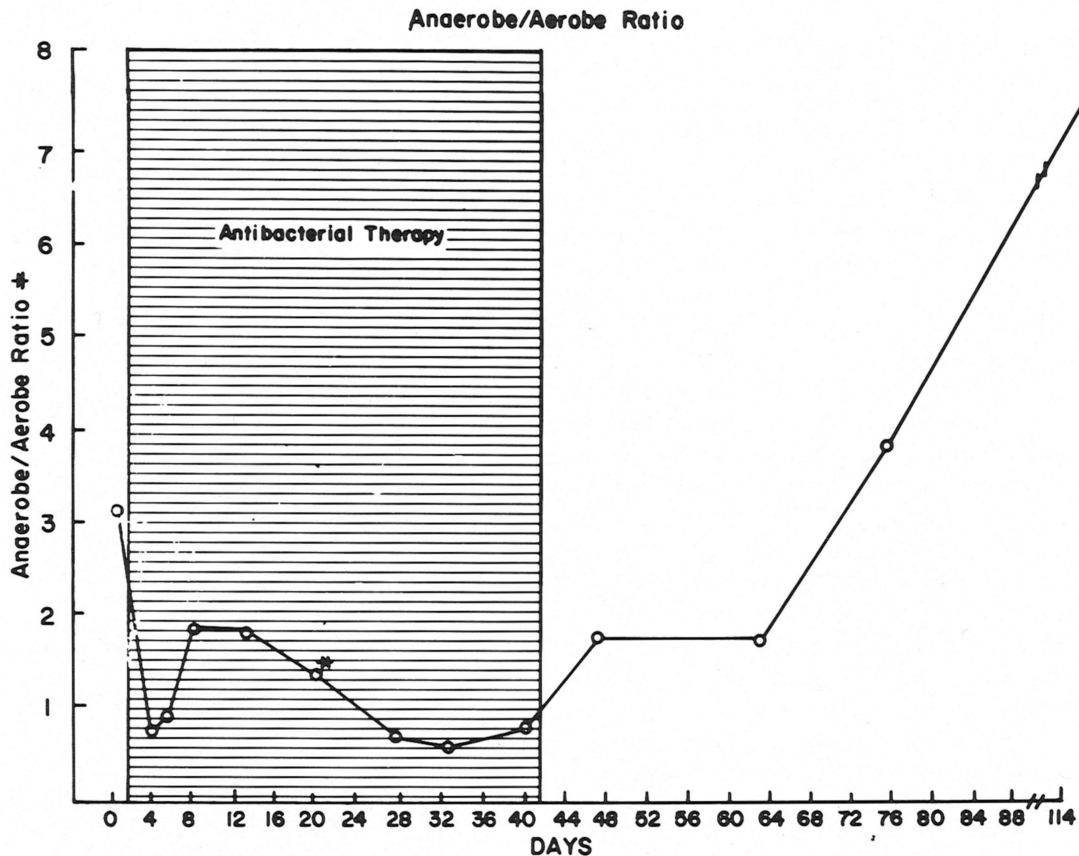

Figure 1. *Anaerobe/aerobe ratio during and after antibacterial therapy. ‡ Ratio of total number of specimens cultured anaerobically to total number of organisms cultured aerobically. * Initial histologic evidence of the keratinization of sulcular epithelium.*

Table 3.
Plaque Bacterial Flora Changes After Cessation of All Antibacterial Therapy

Time*	N†	Total CFU‡ (× 10^5)	Anaerobe/ Aerobe§	*Actinomyces viscosus*	*Actinomyces naeslundii*	*Bacteroides melaninogenicus*‖	*Fusobacterium* Species	*Bacteroides ochraceus*	*Streptococcus sanguis*
days				%	%	%	%	%	%
7	4	5.31 ± 1.23	1.96 ± 0.43	ND¶	ND	ND	ND	1.28 ± 0.64	35.8 ± 8.23
23	4	15.30 ± 6.28	1.88 ± 0.30	ND	ND	ND	ND	8.22 ± 2.08	43.5 ± 7.48
36	2	91.25 ± 46.49	4.08 ± 2.02	ND	2.85 ± 0.25**	ND	12.15 ± 7.45	18.85 ± 0.81	32.7 ± 5.42
74	2	51.75 ± 9.58	7.61 ± 1.37	ND	18.35 ± 11.07	ND	39.90 ± 3.39	9.15 ± 4.35	27.4 ± 2.45

* Number of days after concluding all antibacterial therapy.
† Number of plaque samples cultured.
‡ Total colony forming units (CFU) on a nonselective medium incubated anaerobically, ± standard deviation.
§ Ratio of total CFU of duplicate nonselective plates, one incubated anaerobically and the other aerobically.
‖ Includes three subspecies.
¶ Not detectable; less than 1035 organisms in the total plaque sample, ± standard deviation.
** Percent of total CFU.

and lower levels of *B. melaninogenicus* and *Fusobacterium* species than were observed in the pretreatment samples reported above.[15] A recent study of supragingival plaque in monkeys also noted a low level of *Actinomyces* species (<1.0%).[16] In these quantitative respects, the monkey plaque flora has some resemblance to the flora of the beagle dog.[17]

The shift in the anaerobe/aerobe ratio, as treatment progressed, suggests that selective changes occurred in the flora rather than merely a nonspecific suppression of all organisms. The anaerobe/aerobe ratio appeared to decrease with initial treatment; then successively increase and decrease after Day 13; and finally stabilize, with aerobically grown organisms outnumbering anaerobically grown organisms. This pattern may be attributed to an immediate regrowth of anaerobes which use as nutrients products from tissue breakdown which should be available after scaling. If the flora then was maintained relatively stable by the antibacterial treatment and if the gingiva was allowed to achieve a healthy state, nutrients from the tissue may have been unavailable. Thus, these rather fastidious organisms may have been depleted due to a lack of tissue-provided nutrients.

B. melaninogenicus failed to return to detectable levels up to 70 days after cessation of all antiplaque treatment. After treatment, *Fusobacterium* species increased to a proportional level which, in the pretreatment samples, would have included both that group of organisms and *B. melaninogenicus.* An apparent competitive relationship between *Fusobacterium* and *B. melaninogenicus* is not an uncommon finding in clinical samples.[18]

Recent evidence[19-22] suggests that specific bacteria are associated with periodontal disease. If a causal relationship can be determined, it then becomes practical to direct therapy towards elimination or suppression of specific plaque organisms. Although intensive local therapy or chemotherapy may induce long-lasting changes in the bacterial flora, little evidence[23] exists to support this hypothesis. The present study demonstrates that intensive antibacterial therapy, both local and systemic, produces a major shift in the subgingival flora, and that qualitative and quantitative shift appears to persist at least 74 days after cessation of all treatment.

CONCLUSIONS

1. Daily prophylaxes, systemic tetracycline, and topical chlorhexidine applications produced a marked suppression of the subgingival bacterial flora.
2. Qualitative alterations of the flora appeared prolonged even after cessation of all antibacterial treatment.

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