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

Part II: Inflammation, Mitotic Activity and Keratinization of the Sulcular Epithelium

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THE EFFECT OF an almost complete elimination of bacterial plaque upon the sulcular tissues was studied in Rhesus monkeys. They received thorough scaling and polishing and daily antibacterial therapy for 40 days. Biopsies including the gingival sulcus area were taken periodically during experimentation. They were also taken up to 74 days after therapy was discontinued. Biopsies were evaluated histologically for inflammation and keratinization. Half of each biopsy was exposed *in vitro* to tritiated thymidine to determine mitotic activity of the sulcular and junctional epithelium. The Inflammatory Index decreased from 28.3 in the junctional epithelium (Zone A) and 30.3 in the sulcular epithelium (Zone B) to approximately 3 to 4 in both zones after 40 days of therapy. The Radioactive Index decreased from 24.7 in Zone A and 29.7 in Zone B initially to approximately 2.0 in Zone A and to less than 1.0 in Zone B at Day 40. Both Radioactive Index and Inflammatory Index approached pretreatment levels 74 days after cessation of antibacterial procedures. The most striking feature histologically was a progressive keratinization, demonstrated by specific stains, of the sulcular epithelium. This change was coincident with the decreases in bacteria, inflammation, and mitotic activity. This study achieved an alteration in the sulcular environment by producing a decrease in the number of bacteria, inflammation, and mitotic activity. Keratinization of the gingival sulcus became evident as the sulcular environment changed. It seems reasonable to postulate that reduction in the subgingival bacterial flora reduces inflammation, reduces mitotic activity and allows full differentiation of the sulcular epithelium.

Periodontal disease appears to begin as a bacterial plaque-induced inflammation subjacent to the oral sulcular and junctional epithelium.[1] For this reason the lack of a keratin layer in the gingival sulcus has been interpreted as an inherent weakness in the periodontal structures.

The keratinizing potential of the epithelium has been shown to depend on connective tissue specificity.[2, 3] Since the sulcular epithelium shares the same connective tissue base as the oral epithelium, the sulcular epithelium would be expected to have the genetic determination for keratinization. Studies in monkeys[4] and humans[5] have achieved keratinization of the sulcular epithelium by everting the gingival sulcus lining so that the epithelium was exposed to the oral cavity. In addition, keratinized oral gingival epithelium which was inverted to form a new sulcus lost its keratinization by 21 days.[6]

On the basis of these studies there seems to be little doubt that sulcular epithelium has the potential and predetermination to differentiate into a keratinized epithelium. However, the sulcular environment appears to be a critical factor preventing keratinization of the epithelium. In another publication[7] we have reported an almost complete elimination of the bacterial flora of the gingival sulcus as the result of an intensive antibacterial regime. This report describes changes found in the gingival tissues associated with such an antibacterial alteration in the sulcular environment. Variations in inflammation, mitotic activity, and keratinization will be presented.

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MATERIALS AND METHODS

As previously described,[7] two adult male Rhesus monkeys were subjected to daily antibacterial therapy for 40 days, after having the teeth scaled and polished. The antibacterial regime included: tetracycline hydrochloride IV, rubber cup prophylaxes and chlorhexidine gluconate 0.2% gel topically applied to the gingival sulcus. Plaque samples and biopsies were taken from the buccal aspect of different teeth in both monkeys prior to scaling and at 3, 5, 7, 10, 13, 19, 27, 33, and 40 days of antibacterial treatment. The 33- and 40-day tissue samples were block section biopsies. After all treatment ceased, plaque samples and biopsies were taken after 7, 23, 36 and 74 days. The experimental protocol for the present study already has been published.[7]

Biopsy specimens were removed by means of two vertical releasing incisions and a horizontal incision connecting the apical ends of the vertical incisions. A third vertical incision divided the biopsy. The gingiva was elevated towards the coronal portion and the attachment was dissected from the tooth surface.

Half of each biopsy was fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 6 μ intervals and stained with one of the following: (1) Ehrlich's acid hemotoxylin and eosin, (2) Mallory's trichrome, or (3) Rhodamine B.

The other half of the biopsy was placed on a stainless steel wire grid and processed for *in vitro* tritiated-thymidine labeling.[8] Each specimen was exposed to tritiated thymidine in tissue culture for 24 hours. The specimens were then removed from the culture dishes, rinsed thoroughly in saline solution, and fixed and sectioned as described above for the standard histologic procedures. After sectioning, the radioautographs were prepared using a modification of the technique of Messier and Leblond[9] with an exposure time of 14 days.

Histologic Evaluation

For histologic evaluation the gingival sulcus epithelium was divided into two zones on the basis of accepted morphologic criteria. Zone A included the junctional epithelium which was evident as a thin epithelium with a regular basal cell boundary. Zone B comprised the sulcular epithelium which was thicker than that seen in Zone A, and had an irregular basal cell boundary with some epithelial projections usually present.

A descriptive analysis of the distribution of the keratin layer was performed using the specific stains (Mallory's trichrome and Rhodamine B). In addition, the histologic slides were evaluated for the severity of inflammation in the sulcular connective tissue. An Inflammatory Index (I.I.) was determined for purposes of quantitation.

Inflammatory Index

The Inflammatory Index was calculated as the ratio of inflammatory cells in a microscopic field to the total number of cells in the field. The microscopic field (400×) was positioned so that the basal cell layer divided the field into two approximately equal segments. In Zone A the apical termination of the epithelial attachment was examined. In Zone B the approximate middle of the sulcular epithelium was evaluated.

Radioautographic Evaluation

Radioautographs were evaluated to determine the degree of mitotic activity in the sulcular and junctional epithelium at different time intervals. A Radioactive Index (R.A.I.) was established for quantitation of mitotic activity.

The R.A.I. was determined by counting the labeled and unlabeled cells of the basal layer. The radioactivity of Zone A and Zone B was observed using an oil immersion lens (100×) and was recorded as the ratio of labeled cells to the total number of cells in the counted layer in a microscopic field (100×). Any cell demonstrating more than six intracellular grains was counted as labeled.

Statistical Analysis

Means and standard deviations were computed for R.A.I. and I.I. on the basis of at least nine histologic sections quantitated for each time period. Differences between two time periods were evaluated by means of a paired t test of the null hypothesis that the mean of the difference between data for the two times was zero. The strength of the linear relationship between R.A.I., I.I., and the bacterial anaerobe/aerobe ratio[7] was evaluated by computing the product-moment correlation coefficient, including a t test of the null hypothesis that the population correlation was zero.

RESULTS

Histologic Observations

The time 0 sections exhibited a para- and orthokeratinized oral gingival epithelium, with the keratin stopping abruptly at the crest of the marginal gingiva. The sulcular epithelium had a generally flat basement membrane with gentle undulations. No evidence of keratinization was observed in the sulcular epithelium (Fig. 1). A widespread inflammatory infiltrate was evident approximating the sulcular and junctional epithelium (Fig. 2A).

At 3 to 7 days, the inflammation appeared primarily of a perivascular nature. Rete ridges were prominent in the sulcular epithelium. The keratin layer on the oral surface stopped at the crest of the marginal gingiva (Fig. 2B).

By Days 10 to 13, inflammation was negligible. The dimensions and morphology of the sulcular epithelium were comparable to the oral epithelium. Keratin no longer stopped at the marginal crest but appeared to cover the curve of the crest. Some sections exhibited a long, thin junctional epithelium (Fig. 2C).

From Days 19 to 40, little inflammation was evident

adjacent to the sulcular and junctional epithelia (Fig. 3A). During this period the sulcular epithelium appeared to be covered with parakeratin, with some evidence of orthokeratin in the later biopsies (Fig. 3B).

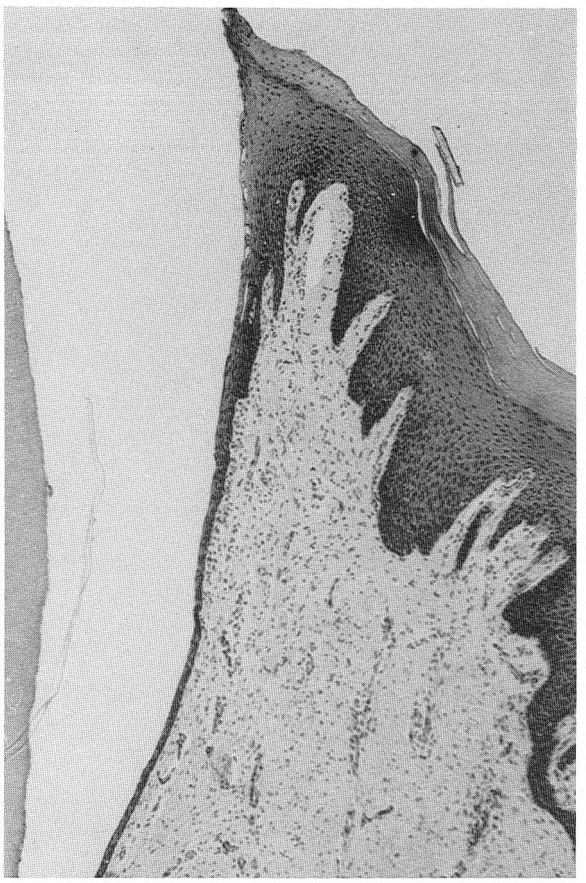

Figure 1. *Normal histology of gingival sulcus of Rhesus monkey prior to any antibacterial treatment (H&E, magnification, × 50).*

Block sections during Day 33–40 revealed keratinization of the entire sulcular epithelium (Figs. 4, 5A).

Seven days after cessation of all treatment inflammation was obvious in all sections, but keratin persisted on the sulcular epithelium (Fig. 5B). After 36 days post-therapy, keratin was evident only on the oral gingival surface and stopped at the crest of the marginal gingiva (Fig. 5C).

Inflammation

The pretreatment I.I. was 28.3 for Zone A and 30.3 for Zone B. Zone A I.I. decreased significantly ($P < 0.01$) with treatment (Table 1, Fig. 6), achieving a level one-third to one-fourth pretreatment values. Great fluctuation was observed in the Zone A I.I. during the experimental period, but by 7 days post-treatment, the I.I. had returned to pretreatment values (Fig. 6).

The I.I. for Zone B was more consistent than for Zone A. The Zone B I.I. decreased sharply (30.3–10.1) in the first week after treatment began. An additional reduction in the Zone B I.I. took place by Day 13, and the I.I. appeared to stabilize at approximately 14% of the pre-treatment level of inflammation. Within 7 days after cessation of treatment the I.I. of Zone B was back to pretreatment values (Table 1, Fig. 7).

Radioactive Index

The R.A.I. was initially 24.7% in Zone A and 29.7% in Zone B. One week after antibacterial therapy began, the R.A.I. for Zone A remained approximately unchanged but then showed a marked decrease and stabilized at approximately 2% for Days 19 to 40. Seven days after cessation of all treatment the R.A.I. for Zone A had returned to 5.0 (Table 2, Fig. 6). Zone B appeared to

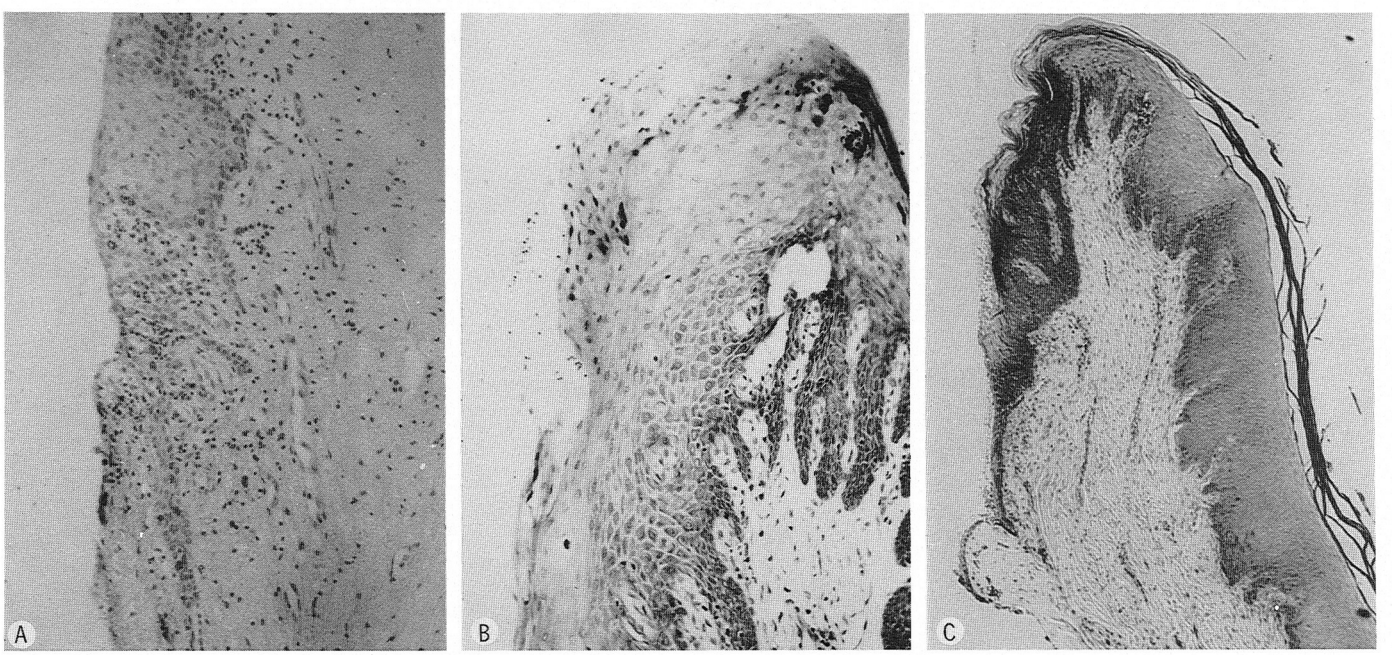

Figure 2A. *Time: 0. Inflammatory infiltrate approximating the junctional epithelium (Rhodamine B, magnification × 100).* **B.** *Time: 5 days. Inflammation is perivascular. Keratin stops at crest of marginal gingiva (Rhodamine B, magnification × 100).* **C.** *Time: 10 days. Keratin covers crest of marginal gingiva (Rhodamine B, magnification × 50).*

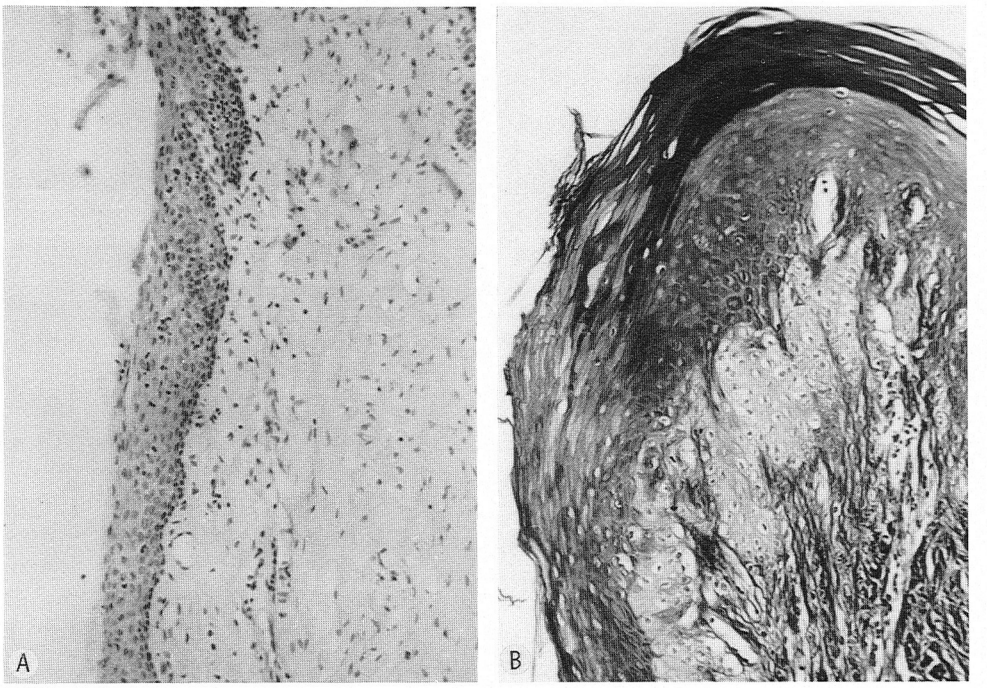

Figure 3A. *Time: 27 days. Negligible inflammation adjacent to junctional epithelium (H&E, magnification × 250).* **B.** *Time: 40 days. Keratin covers sulcular epithelium (Mallory's trichrome stain, magnification × 100).*

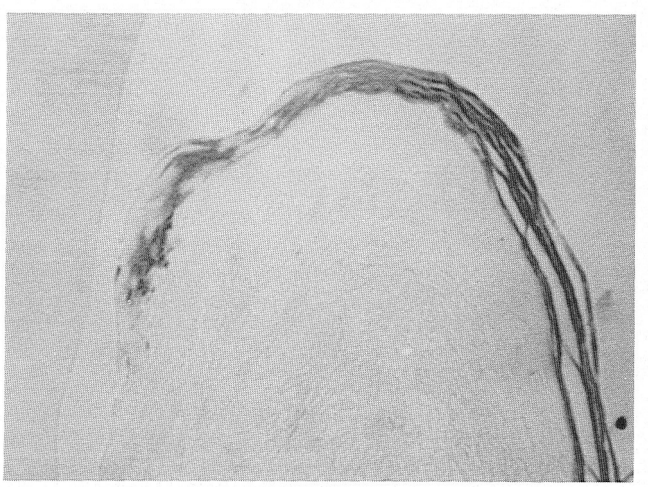

Figure 4. *Time: 40 days. Keratin covers sulcular epithelium (Rhodamine B stain, magnification × 100).*

exhibit a more rapid and consistent drop in the R.A.I. The initial Zone B R.A.I. of 29.7% decreased to 4.1% 1 week after treatment began and thereafter stabilized at approximately 1% or less. Seven days post-treatment the R.A.I. for Zone A had returned to 5.4 (Table 2, Fig. 7).

Correlations

There was a positive correlation between R.A.I. and I.I. for Zone A ($r = 0.376$, $P < 0.05$) and a highly significant correlation for Zone B ($r = 0.850$, $P < 0.001$) (Table 3). Correlations between R.A.I. and the anaerobe/aerobe ratio reported in the previous publication[7] were also highly significant for both Zone A ($r = 0.717$, P 0.001) and Zone B ($r = 0.648$, $P < 0.001$). A positive correlation was present for I.I. and the anaerobe/aerobe ratio for Zone A ($r = 0.424$, $P < 0.01$) and Zone B ($r = 0.607$, $P < 0.001$).

Table 1
Changes in Inflammatory Index

Time*	N†	Zone A	Zone B
days		$\bar{x} \pm SD$	$\bar{x} \pm SD$
0	9	28.3 ± 11.3‡	30.3 ± 13.5
3	9	14.3 ± 4.1	16.9 ± 1.3
5	9	3.8 ± 1.3	10.6 ± 3.8
7	9	7.8 ± 4.2	10.1 ± 4.2
10	9	10.8 ± 5.1	7.4 ± 1.8
13	9	3.7 ± 1.2	4.3 ± 2.1
19	9	7.7 ± 7.4	5.4 ± 5.0
27	9	8.9 ± 2.0	4.6 ± 1.2
33	9	2.8 ± 2.4	2.1 ± 1.8
40	9	3.9 ± 2.1	3.1 ± 1.4
Post-treatment			
7	9	32.5 ± 18.4	28.8 ± 12.5
23	9	40.4 ± 16.5	25.6 ± 12.8
74	9	23.6 ± 8.7	28.1 ± 9.8

* Time 0 samples taken immediately before initiation of antibacterial therapy.

† Number of histologic sections quantitated.

‡ Ratio × 100 of inflammatory cells to total number of cells in a microscopic field (400×) bisected by the basal cell layer of the epithelium.

DISCUSSION

The influence of inflammation on the mitotic activity of the epithelium has been suggested by several studies.[10-12] This study did not attempt to evaluate directly the interrelationship of inflammation and epithelial turnover rate, but inflammation and mitotic activity did appear to change in concert. The findings in this study are in accord with the report of Abrams et al.,[13]

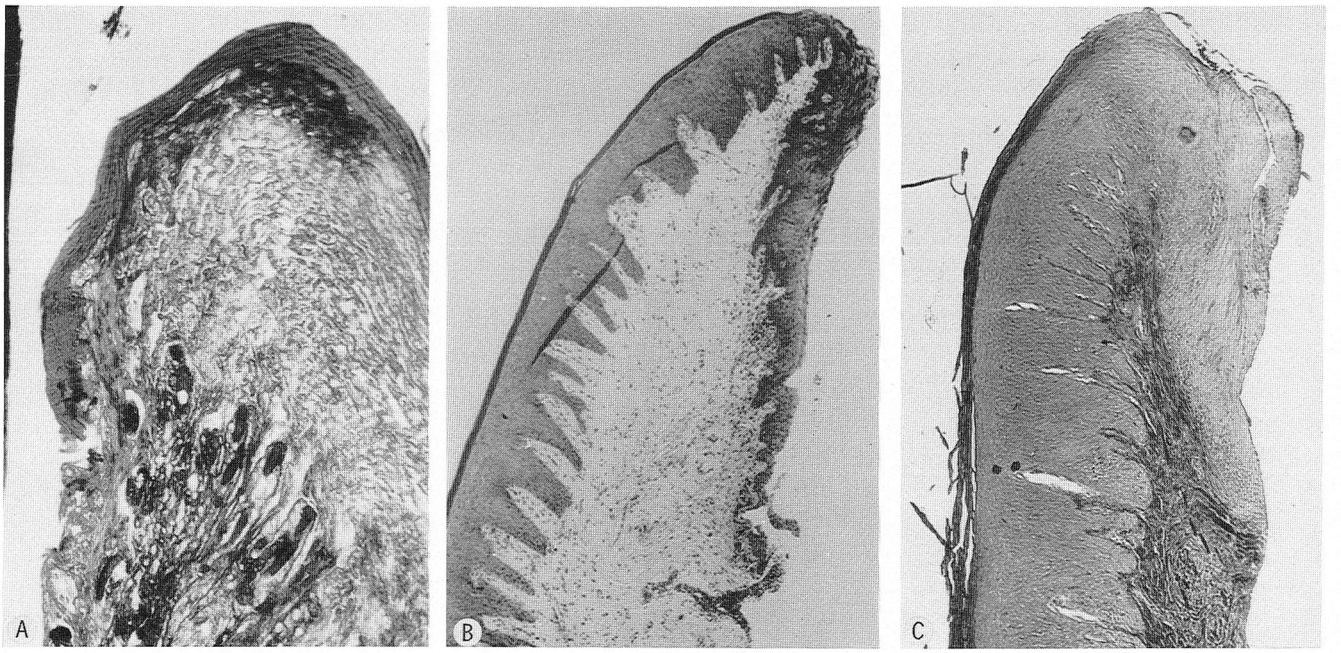

Figure 5A. *Time: 40 days. Thick keratin band covers sulcular epithelium (Mallory's trichrome stain, magnification × 100).* **B.** *Time: 7 days post-treatment. Inflammation is evident but keratin persists in gingival sulcus (Rhodamine B stain, magnification × 50).* **C.** *Time: 36 days post-treatment. Keratin is evident only on the oral gingival surface (Mallory's trichrome stain, magnification × 50).*

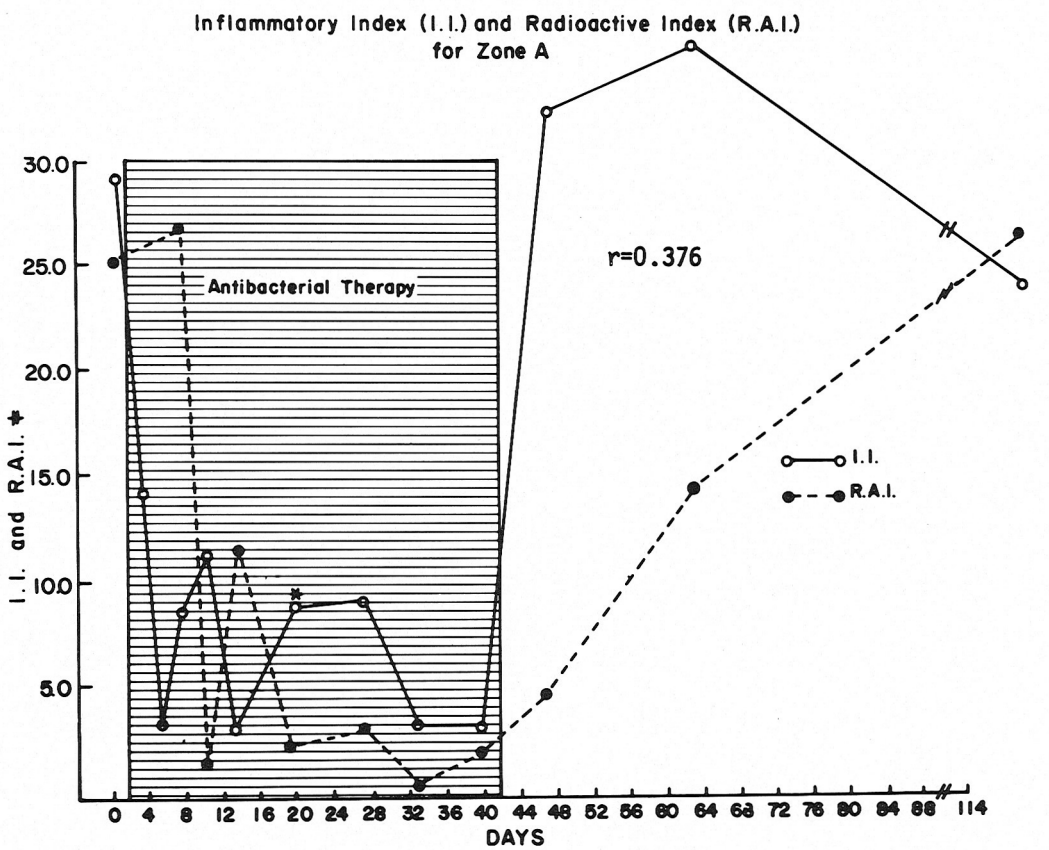

Figure 6. *Inflammatory Index and Radioactive Index for Zone A. ‡ I.I.: Ratio × 100 of inflammatory cells to total number of cells in a microscopic field (× 400). R.A.I.: Ratio × 100 of labeled basal and parabasal cells to the total number of cells in those layers (× 1000 field). * Initial histologic evidence of keratinization of the sulcular epithelium.*

that the presence of a mucosal bacterial flora is associated with an increased turnover rate of the mucosal epithelium on the ileum. In the presence of a viable microbial flora, ileal epithelial cells turned over at a faster rate and reached the surface in less time than had been observed for epithelial cells in germfree mice.

In the present study the I.I. and R.A.I. for Zone A fluctuated somewhat and may represent variability in

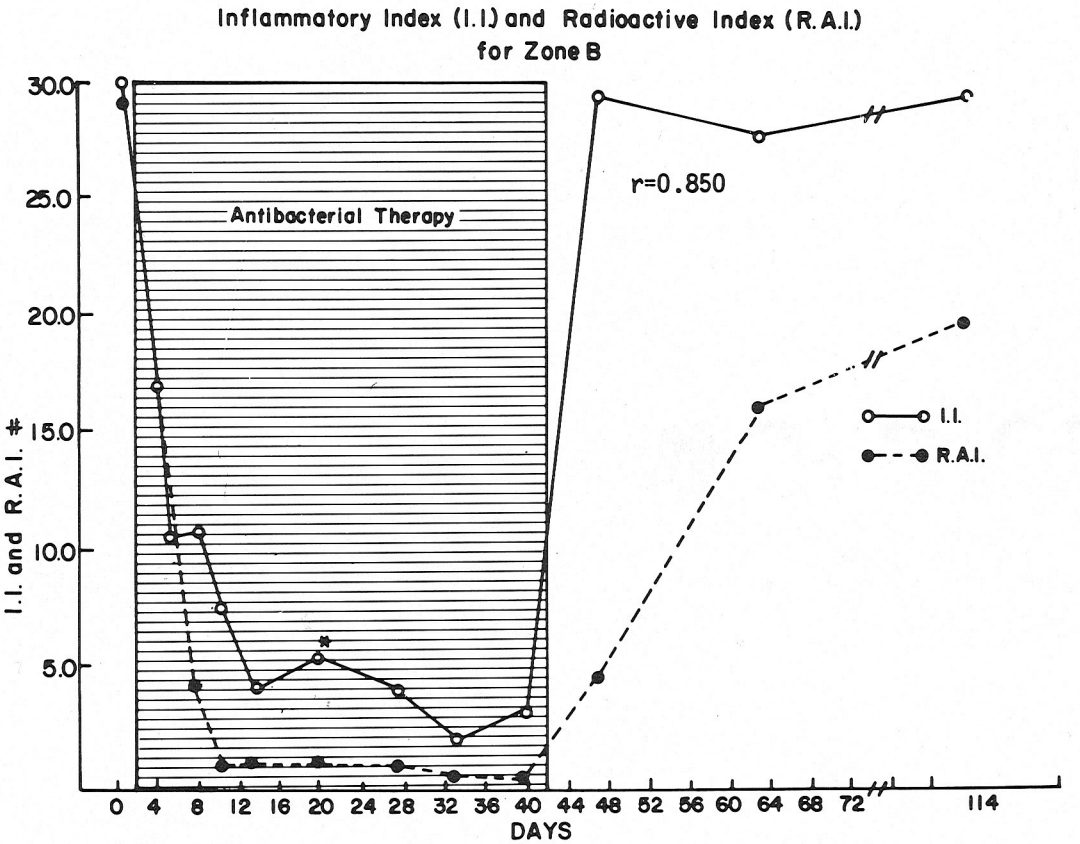

Figure 7. *Inflammatory Index and Radioactive Index for Zone B. ‡ I.I.: Ratio × 100 of inflammatory cells to total number of cells in a microscopic field (× 400). R.A.I.: Ratio × 100 of labeled basal and parabasal cells to the total number of cells in those layers (1000 field). * Initial histologic evidence of keratinization of the sulcular epithelium.*

Table 2
Changes in Radioactive Index

Time*	N†	Zone A	Zone B
days		$\bar{x} \pm SD$	$\bar{x} \pm SD$
0	9	24.7 ± 6.1‡	29.7 ± 8.5
3	NA§		
5	NA		
7	12	26.7 ± 13.0	4.1 ± 3.4
10	9	2.0 ± 2.4	0.7 ± 0.8
13	9	11.7 ± 5.9	1.1 ± 1.5
19	12	2.5 ± 2.1	1.0 ± 1.3
27	9	3.1 ± 2.1	0.8 ± 1.3
33	9	1.0 ± 1.3	0.2 ± 0.5
40	9	2.4 ± 1.6	0.0
Post-treatment			
7	9	5.0 ± 1.8	5.4 ± 2.0
23	9	13.7 ± 6.8	16.7 ± 5.8
74	9	26.9 ± 8.2	19.4 ± 4.3

* Time 0 samples taken immediately before initiation of antibacterial therapy.

† Number of histologic sections quantitated.

‡ The ratio × 100 of labeled basal and parabasal cells to the total number of cells in those layers.

§ Inadequate histologic material available for quantitation.

Table 3
Correlation Coefficients (r) Between R.A.I., I.I., and Anaerobe/Aerobe Ratio Over All Time Periods

Zone	Number of specimens	*r*	Level of significance
	R.A.I. and I.I.		
A	48	0.376	$P < 0.05$
B	48	0.850	$P < 0.01$
	R.A.I. and Anaerobe/Aerobe Ratio		
A	48	0.717	$P < 0.001$
B	48	0.648	$P < 0.001$
	I.I. and Anaerobe/Aerobe Ratio		
A	48	0.424	$P < 0.01$
B	48	0.607	$P < 0.001$

the initial scaling of individual teeth. The close correlation between inflammation and R.A.I. ($r = 0.376$ for Zone A, $r = 0.850$ for Zone B) may have been evident due to the suppression of mitosis below its threshold or maximum value, as suggested by Hopps and Johnson.[14] These correlations were markedly different from correlations reported by Demetriou using similar techniques but without imposing a change in either inflammation or mitotic activity.[15] The correlations found reflect parallel changes in two parameters but, even though highly significant statistically, they allow no real conclusions of a cause and effect relationship.

Since the antibacterial therapy used in the present study is not known to exert direct effects on mitosis, it is assumed that the observed results were mediated through an alteration in the inflammatory status of the gingiva. The antibacterial treatment was effective in reducing the sulcular bacterial load by approximately 10,000-fold[7] and, as a consequence, would be expected to have a

marked effect on the gingival inflammation. In addition, chlorhexidine is known to have direct anti-inflammatory activity.[16] Consequently, there appears to be at least suggestive evidence that reduction of the plaque flora has affected both inflammation and mitotic activity.

It has been postulated that passage of basal cell progeny to the epithelial surface involves a time-dependent maturation process.[17, 18] An alteration in cell turnover rate may affect cell differentiation. Since keratinization requires morphologic and functional cellular changes, an increased passage rate therefore may influence the degree of differentiation achieved by the tissue. According to the findings of Abrams et al.,[13] there is some evidence that the bacterial flora may influence mitotic activity directly or indirectly and affect the differentiation of the epithelium. Besides, the lack of keratinization of some areas of the oral mucosa has been attributed by some to incomplete cell differentiation due to premature desquamation.[19] In the present study, keratinization of the sulcular epithelium was evident coincident with the achievement of the lowest levels of inflammation and mitotic activity.

These findings provide new perspectives on other recent studies. It has been demonstrated that the sulcular epithelium will keratinize when displaced from approximating the tooth.[4, 5] It also has been shown that oral epithelium loses keratinization when positioned to contact the tooth.[6] It seems that the tooth surface or the sulcular environment may influence epithelial differentiation. Since large numbers of bacteria normally inhabit the gingival sulcus, suppression of this microflora appeared to be an appropriate approach toward altering the sulcular environment. The present study demonstrates that an extreme reduction of the subgingival bacterial flora is accompanied by significant decreases in gingival inflammation and mitotic activity of the sulcus. These changes are associated with full differentiation of the epithelium, resulting in keratinization of the sulcular epithelium.

This study was designed to achieve maximum suppression of the subgingival bacterial flora. It is not possible, from the present experimental design, to evaluate the relative influences of the systemic and local antibacterial therapies used.

CONCLUSIONS

1. A significant decrease in sulcular inflammation and mitotic activity was associated with the suppression of the bacterial flora.

2. Keratinization of the sulcular epithelium was achieved concurrent with the above changes in the sulcular environment.

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