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THE ROLE OF SCANNING ELECTRON MICROSCOPY IN PERIODONTAL RESEARCH

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Abstract

During recent years a great amount of research has led to a better understanding of the etiology, pathogenesis and pattern of progression of periodontal diseases. Scanning electron microscopy (SEM) has contributed to this improvement, mainly with respect to histology of periodontal tissues, the description of the morphology and distribution of bacteria on the exposed root surface, analysis of the host-parasite interactions on the gingival pocket wall, and morphological evaluation of root treatment. This review deals with all these topics. Unusual types of SEM research are also described and discussed. Uncommon sample preparation techniques for SEM in periodontal research are described. SEM in periodontal research should be of great application in the near future. Cathodoluminescence, back-scattered emission and immunolabelling techniques will be formidable tools in this field of dentistry.

KEY WORDS: Electron microscopy, periodontal diseases, periodontitis, periodontium, periodontal pocket, dental plaque, scaling, dental.

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Introduction

The periodontal diseases are a family of closely related chronic inflammations characterized by progressive destruction of the tissues supporting the teeth. Typically, relatively short periods of rapid tissue destruction are followed by repair and prolonged periods of remission (128).

The prevalence of these diseases is not known but existing information indicates that their occurrence is very common in non-industrialized countries, and that they afflict a large proportion of adults in the developed countries, thus being a world-wide problem of major proportions (85, 129).

During the last twenty years, basic and clinical investigations in the fields of microbiology, immunology, histology, anatomy and clinical pathology have produced a better understanding of the etiology, pathogenesis and progression of periodontal diseases. New diagnostic tools have been obtained leading to improvement of treatment.

The present review deals with the contributions of scanning electron microscopy (SEM) in periodontology. Most of the pertinent literature will be reviewed. Unusual SEM applications and images will be presented.

General Comments On Periodontal Diseases

It is now widely accepted that bacteria are the most important, if not the only cause of periodontal diseases (84, 127).

From clinical, radiographic and microbiologic characteristics at least four different types of periodontal diseases have been identified: prepuberal periodontitis, juvenile periodontitis, rapidly progressing periodontitis and adult periodontitis (105).

Although more than 300 species of bacteria in the oral cavity are currently recognized, only 5% of these are considered to be strongly associated with periodontitis (39, 84, 87). Actinobacillus (Haemophilus) actinomycetemcomitans, Bacteroides gingivalis, and Bacteroides intermedius are the major suspected pathogens in destructive periodontal disease of the adult (39, 84, 87).

Therefore, the most widely accepted hypothesis is that periodontitis should be considered a specific infection. However, subgingival plaque, associated with periodontal diseases, is very complex and variable, and extreme care must be used in sampling and culturing the microbiota. Furthermore, more than half of subgingival bacteria have not yet been

identified. As a consequence, another point of view is that periodontal disease should be considered, at least in the adult form, to be a non-specific bacterial infection (for a review on non-specific plaque hypothesis see ref. 132).

Bacteria cause periodontal breakdown through virulence factors involved in colonization of the periodontal environments, resistance to host defences and production of tissue damage (76, 126). Also, the host response is important for the outcome of these infections and that periodontal damage or repair depends on the balance between parasite virulence factors and host defences (46). Polymorphoneutrophils (PMNs) are the first line of host defence against bacterial infections. Thus, during these last few years great attention has been focused on the relationship between PMNs and periodontitis (for review see ref. 26, 95, 134).

It was recently discovered that prepuberal (105, 106), juvenile (34, 133), and rapidly progressing periodontitis (32) are frequently associated with defective function of PMNs. Most types of periodontitis affect a limited number of teeth in the dentition and they advance with episodes of activity followed by periods of remission or repair. At a given time, as little as 1 to 10% of deep periodontal pockets may go on to additional breakdown (128). In spite of the enormous amount of information gathered about the etiology, pathogenesis and the pattern of progression of periodontitis in the past 15 years, the treatment of these chronic inflammatory diseases is basically the same as at the beginning of this century. The main goal of periodontal treatment is to establish conditions which will allow daily non-specific removal of the supra- and subgingival plaques (112). This can be achieved by scaling root planning (with or without periodontal surgery) and a strict and rigorous program of home-care procedures for oral hygiene. The fact that few, if any, other infections can be treated only by improved hygiene should warn us that current therapy may not yet be optimal.

Sample Preparation and Related Problems

The periodontium, properly described as the supporting apparatus of the tooth, is a highly organized structure composed of several tissues. While dental cementum and alveolar bone are mineralized, periodontal ligament and gingiva are typical soft tissues. Crevicular fluid and bacterial plaque, constantly associated with periodontal lesions, have a high water content whereas the calculus is strongly mineralized. As a consequence, different sample preparation techniques must be used for different structures. Techniques for processing specimens for SEM investigation of periodontal tissues are basically the same as those adopted in other fields of biological investigation. Comprehensive reviews are available that discuss in detail the usual methods of specimen preparation for SEM (11 - 15). Here we describe a few of the uncommon methods of sample preparation that have interesting applications in periodontology.

One might be interested in studying the structure of alveolar bone, cementum and calculus which have been stripped of virtually everything except their mineral component, referred to as anorganic. On the other hand, our interest could be focused on the organic coatings that cover the root, on the cellular component of the bone or on the study of soft tissues or crevicular fluid. We will call these samples organic. From a general point of view, two basic procedures are available (15). If we want to look at the anorganic morphology of bone, cementum and calculus, we can deproteinize the samples with a fresh solution of NaOCl (sodium hypochlorite), widely available as household bleach. Concentrated NaOCl solutions are 14% and these may be diluted 1:1 with water to obtain a less corrosive agent. Careful and plentiful washing in distilled water is recommended to remove sodium chloride crystals, if any, from the sample surface. No fixation is required for anorganic samples to be prepared.

For organic samples, fixing should be at physiological temperature and pH, and the osmotic pressure derived from the non-fixative components of the mixture should match that of normal extracellular fluid. Hypotonic fixation leads to swelling, hypertonic fixation to shrinkage (13). Primary fixation is usually performed with a solution of 1-3% glutaraldehyde in phosphate or, better, in cacodylate buffer which avoids precipitation of salt during fixation. Post-fixation with osmium is not always required (88), glutaraldehyde alone works just as well (136).

Although the improvement in electron microscopic techniques during the past 15 years has enabled us to obtain good biological specimens for SEM investigation, shrinkage and distortion due to dehydration remains an unresolved problem. Swelling of animal tissues occurs in concentrations less than 60% by volume of all the common dehydrating solvents and shrinkage occurs in 80% ethanol or acetone, so that the specimen is reduced to about 20% below its original volume. Additional shrinkage occurs in 90 and 100% ethanol, and during critical point drying (CPD) (16, 19).

Information regarding the mineralizing front of cementum in periodontal health and disease is easily obtained by deorganifying the root surface as described above. The common reported artefact in this procedure is the superficial cracking of cementum due to dehydration. This artefact is easily identified by its typical appearance and, unless optimal SEM surface morphology is required, can be avoided by using the replica technique, thus studying the specimen indirectly (29).

The replica technique has rarely been used in periodontology. Useful information was gained by this technique about the topographical changes which follow a root treatment (31). Comparisons of the same root area before and after root instrumentation has been recently performed with the replica technique (31). The materials most commonly used to obtain replica are a silicone impression material which is used to make the negative impression, and an epoxy resin, used to re-establish the positive structure (12, 13, 15, 107, 109, 119). This technique was first described by Grundy (52) and subsequently elaborated by Barnes (6). Systematic investigations of test materials for negative-positive replica combination for SEM have recently been published (22,28).

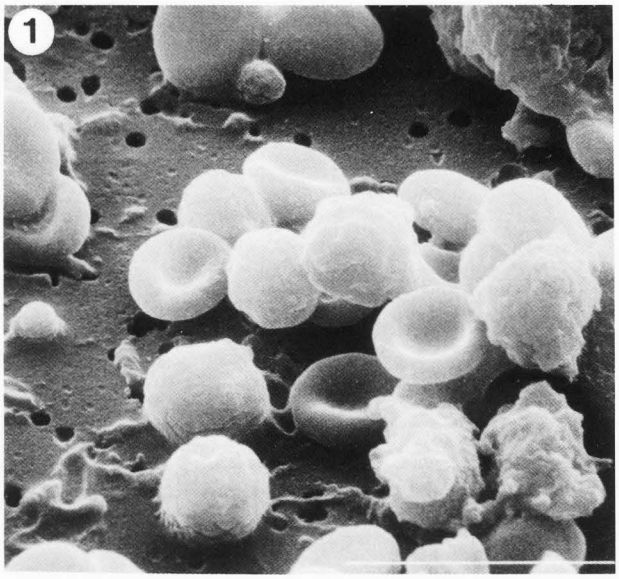
Briefly, a silicone rubber impression material (in our laboratory we routinely use Xantopren Plus ®, Bayer) is applied to the tissue and removed by gentle peeling after the setting of the material. A resin impression (in our experience "Scutan" ® Espe gives the best results) of the negative replica provides a positive model with the same topography as the original surface. The positive model is coated with 20

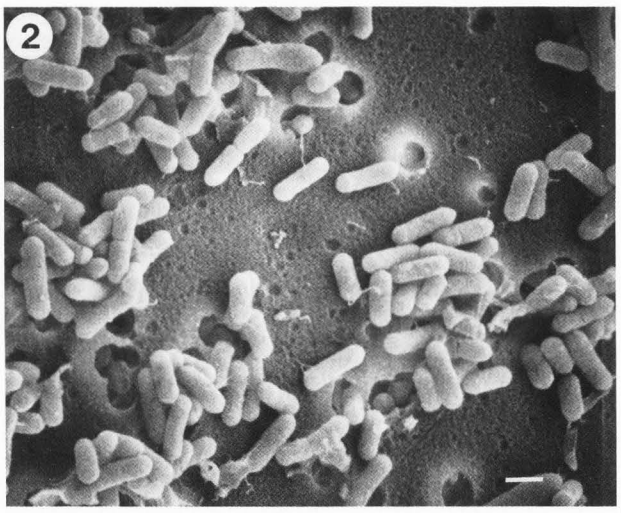
nm of gold-palladium and examined under SEM in the secondary mode. Both the silicone and the epoxy resin have to be applied to the surface to be investigated in small quantities and under gentle air current. Pitting and bubbling, easily recognizable, are the most common artifacts in the replica technique (50). Recently a new replica technique has been developed, using a low viscosity impression material that is subsequently copper plated and filled with synthetic plaster (73). However, in our laboratory, we were unable to see any advantages of this technique over the more common and less expensive traditional technique.

Crevicular fluid can be obtained from individual sites (117) by modifying the method originally proposed by Skapsky and Lehner (122) for immunological investigations. The area of interest is isolated with cotton rolls and gently dried with an air syringe. Then, the needle of a 50 µl microsyringe is placed just coronal to the interdental papilla of the tooth surface and 10 µl Hanks' balanced salt solution are ejected and reaspirated repeatedly. The crevicular fluid wash obtained is transferred to a 1 ml syringe filled with 2.5% glutaraldehyde and fixed for 30 min. The solution is filtered through a Nucleopore-polycarbonate filter. The filter is then dehydrated in increasing concentrations of ethanol, dried by CPD, sputter-coated, and studied in the SEM (Fig. 1). With this technique one can study the cellular component of the crevicular fluid (i.e., leukocytes) and analyze the microorganisms forming at the outside layer of the subgingival plaque. A similar technique can be used to study the morphology of bacteria in pure cultures (Fig. 2) (70).

Several SEM investigations have dealt with plaque distribution and composition (27, 30, 33, 60, 67, 98, 113). Usually, immediately after extraction, the tooth is gently rinsed in tap water to remove all loose debris. While tooth washing after the surgical procedure removes blood and saliva from the root surface, it probably disturbs the outer bacterial layers of the plaque. In our experience the tooth need not be washed after the extraction to study the bacterial plaque. A papillary injection of local anesthetic just prior to the extraction will minimize the bleeding. Saliva coating does not represent a problem studying the subgingival plaque. Artifacts of the distribution of the microorganisms on the root surface can arise both during the luxation of tooth and during its removal from the socket.

Investigations in periodontology by SEM have usually dealt with naturally exposed surfaces. Any form of cutting can cause plastic deformation of the surface layers of the tissue and unless one is interested in the inside of cavities so exposed (i.e., lacunae in alveolar bone), it is better to avoid the use of cutting blades near cells that are to be examined. Some recent SEM investigations have demonstrated that bacteria can invade the gingiva of the pocket





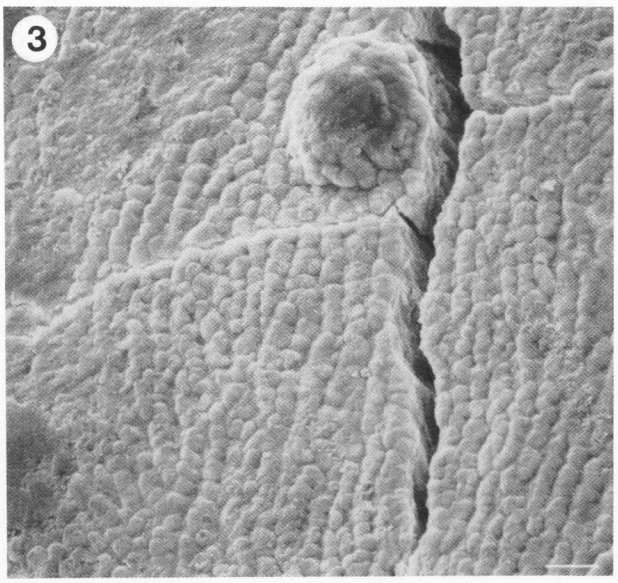


Fig. 1. Sample of crevicular fluid obtained by a modification of the technique of Skapsky and Lehner. Bar = 10 µm.

Fig. 2. SEM appearance of Bacteroides gingivalis. Bar = 1 µm.

Fig. 3. The low rounded mounds are Sharpey's fibers. A cementicle is seen in the upper part of the image. The surface cracking is an artefact of dehydration. Bar = 10 µm.

wall in juvenile periodontitis (25) and in the deep pocket of patients with advanced chronic periodontitis (116). In these studies the inner gingival surface was exposed by cutting with a surgical scalpel. The microorganisms detected, in both epithelium and connective tissue may be an artefact due to the carrying inside during the cutting procedure of bacteria usually present on the surface of the gingiva. Freeze-fracturing the specimen is the optimal method for exposing the deep surface of a given sample (12, 15). In spite of the simplicity of this procedure, to our knowledge, freeze-fracturing has never been used with SEM for periodontal investigations.

SEM and Anorganic Root Surface

Cementum is a calcified bone-like substance that covers the root of the tooth and provides attachment or anchorage for the periodontal fibers. Several researchers have performed both light and transmission electron microscope (TEM) studies, but these studies have been limited to sections.

SEM has made it possible to gain detailed information about the natural surface of cementum. A fundamental increment to knowledge in this field has been contributed by Boyde and Jones (10, 17, 64, 65, 75). They have extensively investigated the morphology of cementum surface in both humans and other mammals. The surface of the root after normal extraction procedures is usually obscured by a dense matrix of collagen fiber remnants of periodontal ligament. Boyde and Joned obtained more useful information from surfaces of specimens rendered anorganic by treatment with either hot 1,2-ethane diamine in a soxhlet apparatus or a cold 5% solution of NaOCl, followed by washing in water. The SEM aspect of anorganic cementum is characterized by the position of the Sharpey fibers, generally present in one or both of two distinct ways: either they appear as projections above the general plane of the mineralizing front (Fig. 3) or as a depression in this front. The projections, low rounded mounds, represent Sharpey fibers that are mineralized to a degree beyond that of the intrinsic fibers, whereas the depressions represent the site of Sharpey fibers that are not mineralized to the same degree as the intrinsic fibers. Depressions are considered typical of an actively forming cementum and projections typical of an acellular, resting cementum.

Areas of resorption have been identified on the basis of the excavated shape of "Howship's lacunae" in the apical area of the roots in young teeth. Recently, in a SEM investigation of 10 teeth extracted from two patients with juvenile periodontitis and rapidly progressing periodontitis, it was proposed that such lacunae, when present in periodontally involved teeth, could act as natural sites for sheltering and retaining subgingival plaque, even after root planing, thus causing recurrent periodontitis (118). It is substantially documented that single or grouped areas of root resorption can occur in caries-free and periodontally healthy teeth (53, 54, 92) and as a result of some periodontal surgical techniques (68, 89, 105). One of the most interesting studies of root resorption was performed by Henry and Weinmann (55), using the light microscope. They studied 261 teeth from 15 autoptic human dentitions, cut on bucco-lingual and mesio-distal planes and stained with hematoxylin-eosin. The results showed that: 1) root resorption must be considered a para-physiologic event, 2) the number of root resorptions was correlated with age, 3) the width of a single root resorption is a good index of severity of lesion, whereas the number of root resorptions is related to the number of traumas that caused the lesion, 4) root resorptions are usually located on the apical third of the root.

The root resorption of periodontally involved teeth has been studied by SEM (7, 64, 72). Unfortunately, no attempt has been made to correlate the presence and distribution of root resorption with the amount of periodontal damage. In order to assess the prevalence, the distribution of root resorption areas and their correlation with certain periodontal indices (plaque index, bleeding index, probing depth), we recently studied 64 teeth extracted for periodontal disease and 28 teeth extracted for orthodontic reasons (Carrassi, Abati, Agliati, unpublished). Before the surgical procedure, the periodontal index and clinical history were carefully recorded. After the extraction, a notch was made along the insertion of the epithelium-connective attachment, rendered visible by immersion in a solution containing 1% toluidine to differentiate exposed and non-exposed root. Then the teeth were rendered anorganic with a 7% NaOCl solution and processed for SEM. The results showed that periodontally involved teeth are constantly associated with areas of root resorption. Further, the number and the width of the defects was significantly correlated with the severity of loss of attachment. Lacunae in periodontally healthy teeth were usually small, superficial and grouped (Fig. 4), whereas resorption on periodontally involved teeth were larger, deeper and scattered. As revealed by the notch, lacunae were usually under the soft tissue attachment and in the third apical area of the root (Fig. 5). Both cementum and dentine can be involved by root resorption (Figs. 6, 7). A possible explanation of these findings is that areas of resorption may be related to occlusal function. The augmented tooth motility could explain the elevated presence of resorption in periodontally involved teeth.

The typical morphology of cementum can be altered in particular conditions, such as juvenile periodontitis, Lindskog and Blomlof (80), studying 4 molars extracted from 4 patients with juvenile periodontitis, found extensive areas of cementum hypoplasia with exposed dentinal tubules. They suggested that the development of disease is initiated by a hereditary development disturbance of the cementum.

Fig. 4. Several resorption lacunae in the apical third of a tooth extracted for orthodontic reasons.

Fig. 5. A wide resorption lacuna in the apical area of a periodontally involved tooth.

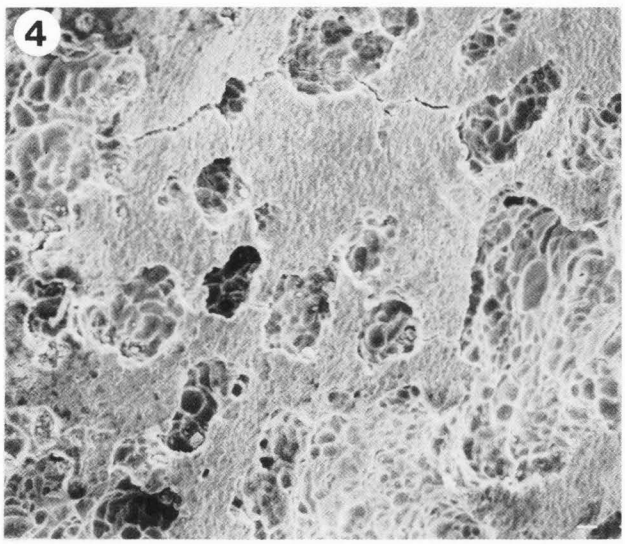
Fig. 6. Typical feature of Howship's lacuna. The resorption is limited to the cementum.

Fig. 7. In this case a more severe resorption involves the dentine. Peritubular dentine is clearly seen.

Fig. 8. Calculus on the root surface. The bacterial matrix is calcified showing cocci and rods.

Fig. 9. the extrabacterial matrix is calcified. The shadows of cocci, rods and spirochetes are shown.

Bars = 10 μm (Figs. 4 - 7); and = 1 μm (Figs. 8, 9).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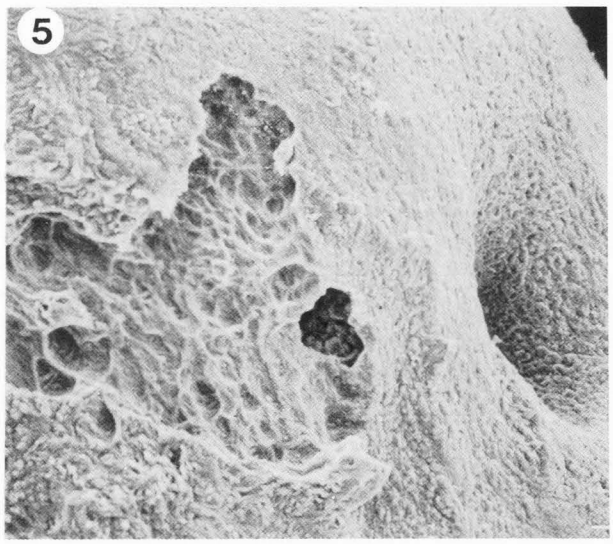
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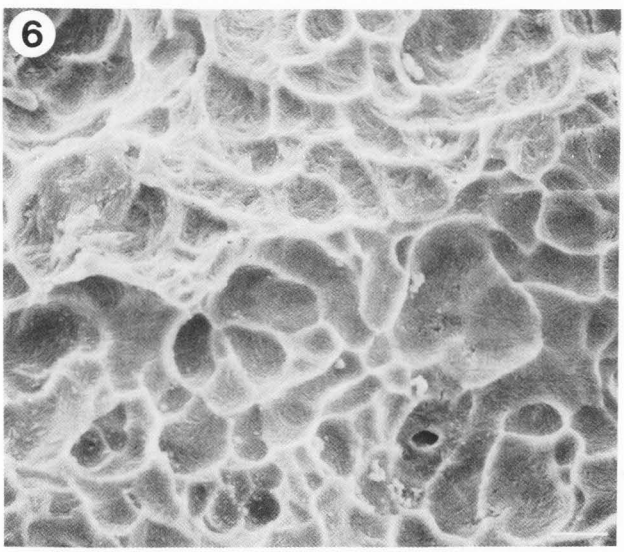
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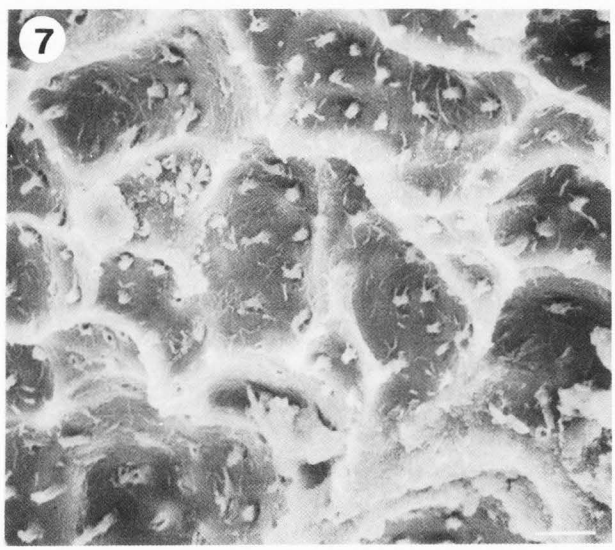
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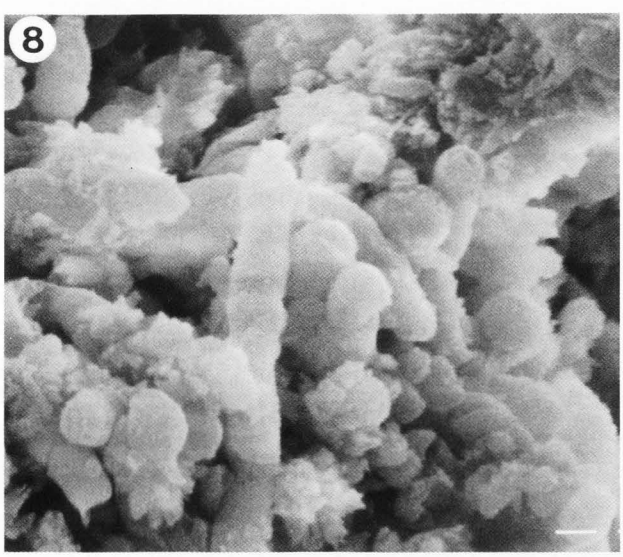
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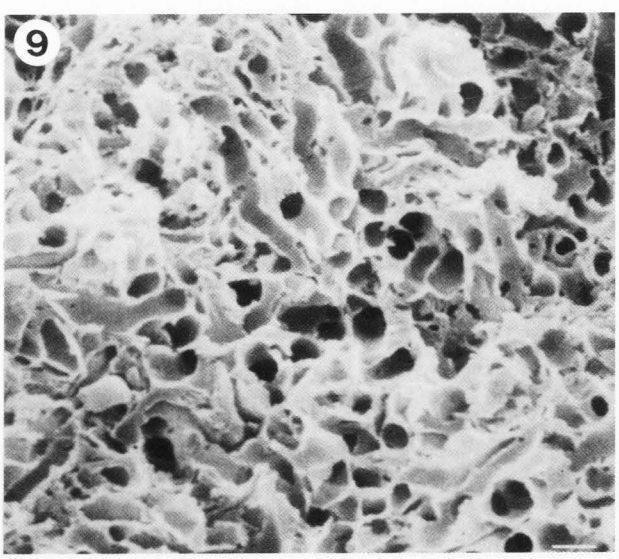
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It is evident that SEM investigation of the morphology of the cementum surface in health or in disease can be useful. SEM is the only instrument that at present enables one to examine the whole surface of the root at high magnification.

Calculus, which may be defined as calcified or calcifying deposits on the teeth, is a plaque that has been mineralized. It is usually covered by a layer of plaque which "in vitro" could be removed by an organic solvent to reveal its morphological appearance. The distribution and structure of calculus have been studied by light microscopy and TEM (for review see ref. 91). However, these studies were limited by the small areas that can be studied.

The first SEM description of the morphology of calculus formation and distribution on human teeth was provided by Jones (62, 63), who described two basic patterns of mineralization of the plaque (Figs. 8, 9): a globular or "calcospheritic" pattern, and a creeping pattern. Mineralization of the intermicrobial matrix usually preceded that of microbes, but occasionally the bacteria led the mineralization front in some areas. Fractured specimens of calculus revealed two basic structures. One type is continuously mineralized calculus surrounding vertically aligned filaments or rods. In the other, incompletely mineralized, a much more irregular and varied calculus still contains unfused or partly fused mineral clusters throughout its depth. Further information about calculus morphology obtained by SEM has been provided by Eide et al. (40, 41). In a recent comprehensive review of calculus attachment by Canis et al. (24) studied 63 extracted teeth, and provided SEM evidence of a cuticular attachment of calculus matrix to tooth surface. The most frequently encountered method of attachment was apparent molding of calculus matrix onto the surface of cementum.

SEM and Dental Plaque

The root of periodontally involved teeth is covered by a thick layer of bacterial plaque and may undergo changes in its histological appearance, and its physical, chemical, and immunochemical properties (83). Bacterial plaque has been extensively investigated to identify bacteria which colonize the exposed root surface and the association between certain microorganism and periodontal health or disease (51, 87, 96, 100, 127, 131). At present there is a general agreement that no more than 6-12 bacteria are commonly associated with periodontitis (97, 127). However, in spite of the overwhelming evidence of the strong association between certain bacteria and periodontal diseases, definite proof that such diseases are specific infections is lacking.

SEM investigation of the structure and composition of dental plaque were first published in 1971 (60). A heterogeneous distribution of bacterial forms along the root and a typical association of coccoid forms and filamentous bacteria (probably Bacterionema matruchotii) have been revealed (61) (Fig. 10). More detailed information about the relationship between the most apically located plaque and the epithelial attachment has also been obtained (113).

Recently, the morphology of subgingival plaque in Papillon-Lefevre syndrome (67), in juvenile periodontitis (33), in chronic adult periodontitis (98), and in rapidly progressing periodontitis (27, 30) have been investigated by SEM. As a consequence of this research, it has become clear that bacteria are highly organized on the root surface. In the upper and in the middle zones of the root, cocci and filaments are the bacteria most frequently identified. The cocci often show the well known "corn-cob" feature. The most apically located plaque, which from a theoretical point of view is located where it can exert the most detrimental effects on the host, has been described as formed principally by small and medium spirochetes and straight and curve rods (27, 30, 98) (Fig. 11).

Occasionally, bacterial "microcolony" formation has been seen by SEM. The small size of the microorganisms suggested that they might be species of the genus Mycoplasma (25). The term "pioneer bacteria" has been proposed for the most apically located microorganisms on the advancing front of subgingival plaque (113). They are rods and to a lesser extent spirochetes (Fig. 12). One of the most impressive findings about subgingival bacteria is the absence of coccoid forms in the apical third of the subgingival plaque. This might be explained by the antagonism known to exist between some cocci and certain rods considered periodontopathogens (56). Cocci play a pivotal role in the colonization of bacterial plaque on the root surface (135).

Early phases of bacterial colonization on human cementum "in vivo" have been recently studied (1). Slabs of cementum were obtained from sound teeth extracted for orthodontic reasons and rendered anorganic with 7% NaOCl solution. The cementum slabs were glued to orthodontic brackets and positioned on the upper canines, premolars and first molars of 8 volunteers. Then, the brackets were removed after 2, 4, 8 and 24 h, dehydrated with graded alcohols and critical-point dried with CO_2. The samples were finally mounted on an aluminium stub, sputter-coated with 20 nm of gold-palladium and observed in a SEM operating at 15 kV in the secondary mode. A thin pellicle covered the cementum surface after 2 h and a few microorganisms were found. After 8 and 24 h a thick layer of coccoid which formed in an amorphous and fibrillar extrabacterial matrix was noted (Fig. 13). Filaments inserted perpendicularly into the plaque were also detected after 8 h, and more commonly after 24 h (Fig. 14). Early bacterial colonization is a selective process, mediated by an organic pellicle and mainly involving a coccoid population.

There are two important limitations of SEM in investigation of subgingival plaque. It is impossible to differentiate between dead and live bacteria and microorganisms can be classified only morphologically. An important advance in this field would be the addition of immunolabelling techniques to SEM investigation of subgingival plaque.

SEM and Gingival Pocket Wall

It is now generally accepted that in certain cases bacterial invasion into gingival tissue may occur. This important finding was first clearly demonstrated by Listgarten (82). With TEM it was possible to see superficial (300 μm) spirochete invasion within periodontal tissue in acute necrotizing gingivitis. Following this report, further studies by TEM documented bacterial invasion in juvenile periodontitis (47), and in very advanced periodontitis (44). The invading microorganisms were morphologically heterogeneous and mainly gram-negative, including coccal,

bacillary, filamentous and spirochetal forms. Recently, yeasts have been seen by SEM in juvenile periodontitis (49).

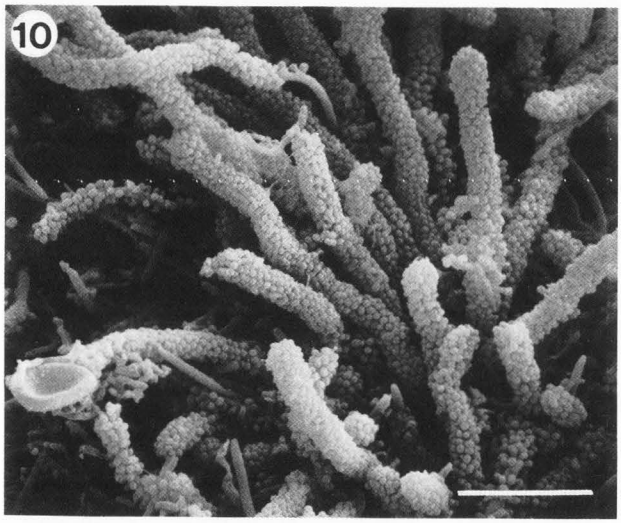
Fig. 10. "Corn-cob" bacteria at the surface of the root. Bar = 10 μm.

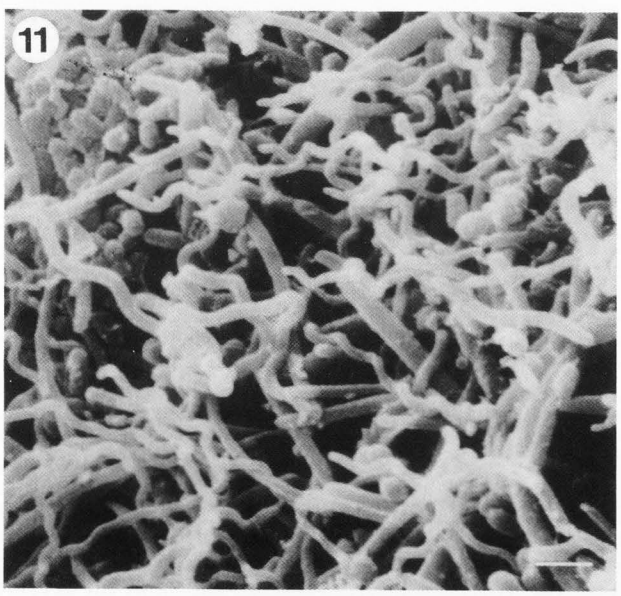
Fig. 11. Morphology of subgingival plaque on the most apically located root area in a case of Rapidly Progressing Periodontitis. Bar = 1 μm.

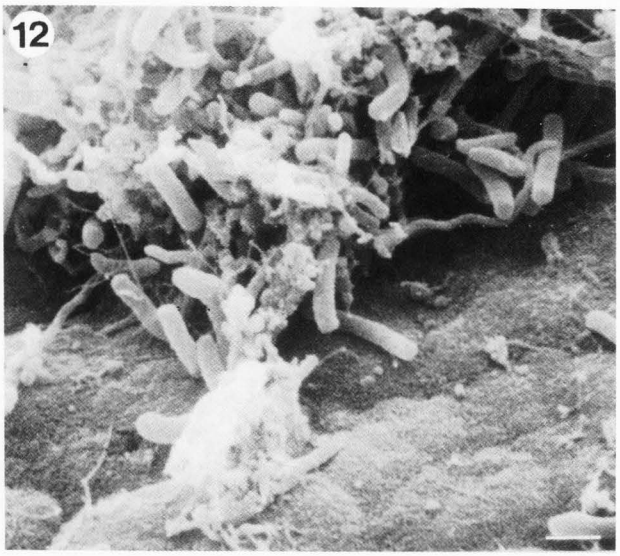
Fig. 12. "Pioneer" bacteria on the advancing front of the plaque. The underlined microorganism has a morphology similar to that described for Bacteroides genus. Bar = 1 μm.

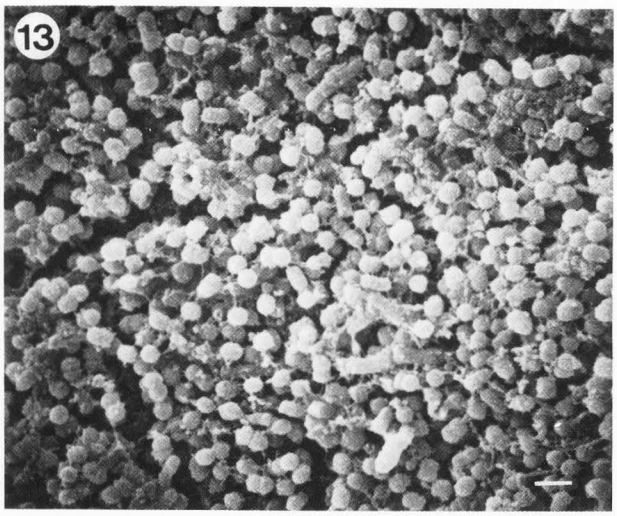
Fig. 13. Experimental plaque colonization, 8 h period. Cocci and rods cover the sample surface. Bar = 1 μm.

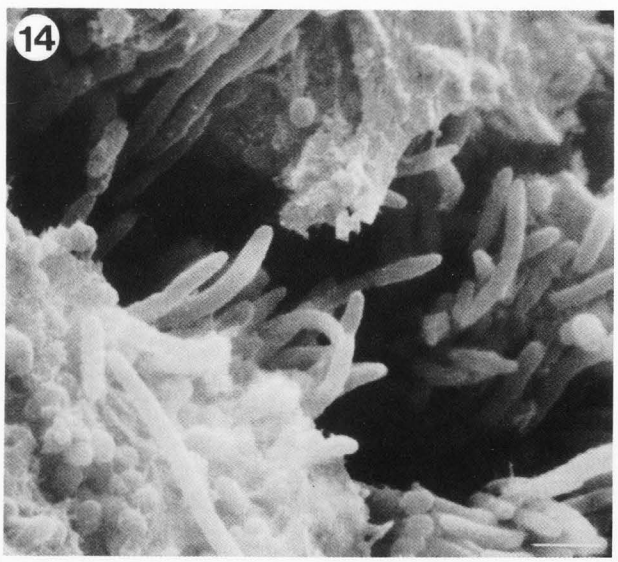
Fig. 14. Experimental plaque colonization, 24 h period. Filaments and long rods inserted perpendicularly to the plaque surface. Bar = 1 μm.

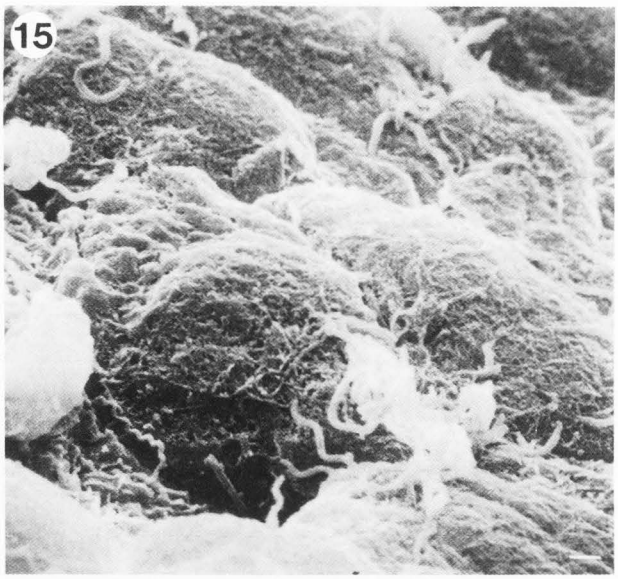


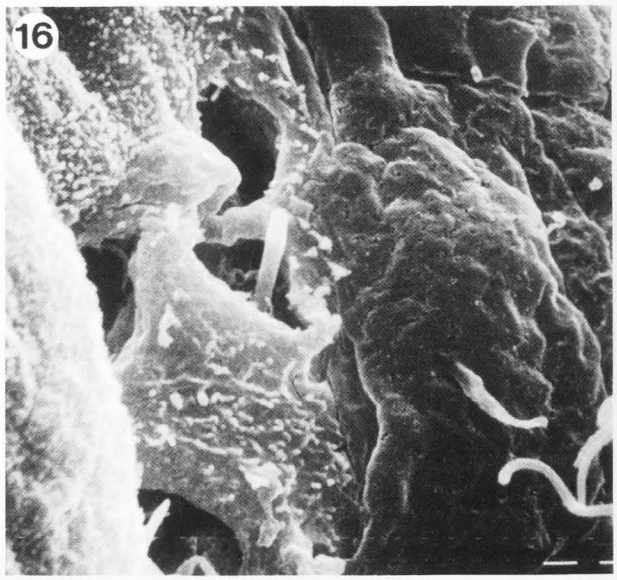


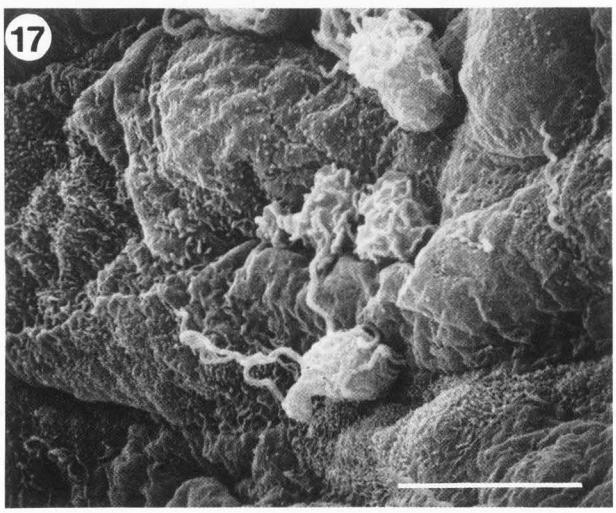


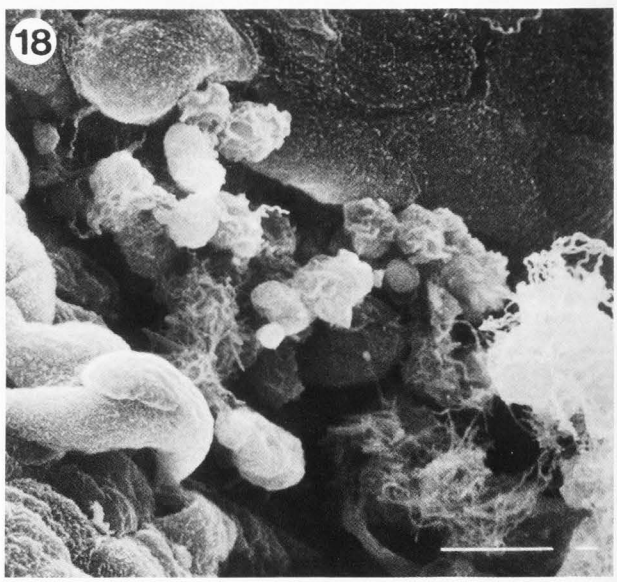












Figs. 15 - 18. Pocket wall of a patient with R.P.P. Spirochetes entering into an epithelial ulcer (**Fig. 15**); and entering into an intercellular space (**Fig. 16**). **Fig. 17.** Several PMNs on the gingival surface. One of these is phagocytizing two spirochetes. **Fig. 18.** Leukocyte exocytosis from a wide epithelial ulcer.

Bars = 1 μm (Figs. 15, 16); and = 10 μm (Figs. 17, 18).

SEM has been used for the morphological description of the gingival pocket wall and the detection of bacterial cells within gingival tissues, mainly in the contributions of Saglie et al (115, 116). SEM evidence of bacterial invasion in localized juvenile periodontitis (25) and in advanced periodontitis in humans has been obtained (116). In five of 8 cases of advanced periodontitis, bacterial penetration into the epithelium was found, in one of these five cases bacteria had reached the connective tissue. Several bacterial morphotypes were identified within the enlarged intracellular spaces of the pocket's epithelial surface: cocci, short rods, filaments and spirochetes (116). In one case of juvenile periodontitis, invading bacteria were mainly gram-negative fusiforms, coccobacilli and spirochetes (25). The microorganisms appeared to have invaded the epithelium and underlying connective tissue, reaching the bone surface.

Microorganisms identified as Mycoplasma were also found in some areas (25). Several papers have pointed out that the presence of bacteria in tissue may be due to artificial introduction during collection of the sample or during its processing (9, 48, 137). Although the research described above has shown intercellular bacterial location and a definite pattern of penetration, the possibility of an artefact

cannot be ruled out. In advanced periodontitis, the morphology of the natural surface of the pocket wall has been visualized by SEM (114, 115), such as in rapidly progressive periodontitis (32). Several morphological features have been found on the gingival wall and have been described (116): areas with epithelial desquamation, areas with leukocyte bacterial interaction, areas with emerging leukocyte, areas with bacterial accumulation and areas with ulceration. Rapidly progressive periodontitis (R.P.P.) is a relatively rare clinical entity characterized mainly by severe and diffuse periodontal damage, age of onset between puberty and age 30 and frequent functional defects of neutrophils and/or monocytes (104). The morphology of the gingival pocket wall of a group of patients with R.P.P. has recently been investigated by SEM and TEM (32). Four untreated patients with R.P.P. (17-32 years of age, mean 22.4) were selected for this study. After informed consent was obtained from the patients, two gingival biopsies of pocket wall were taken from each patient and processed for SEM and TEM. By TEM no evidence of bacterial invasion was found in gingival sections, although SEM occasionally documented the penetration of spirochetes into the superficial epithelium (Figs.15, 16). Areas of exocytosis were evident on the samples examined by SEM. Spirochetes in contact at one end with the neutrophil surface are often seen. This feature is considered to indicate an initial phase of the phagocytic process (Figs.17, 18) (32).

The concept that repeated episodes of tissue invasion by bacterial pathogens might explain the episodic nature of periodontitis is an attractive one. Unfortunately, with the possible exceptions of acute necrotizing gingivitis and juvenile periodontitis, there is no reliable evidence that this is, indeed, the case.

SEM and Root Cleaning Instruments

Cleaning the root surface by removal of dental plaque and calculus is the most important phase of periodontal treatment (79). The SEM allows direct examination of the whole tooth surface and combines high resolution with great depth of focus. Therefore, it is not surprising that a great number of studies dealing with the effects of various instrumental techniques on the root surface have been conducted with SEM (4, 31, 37, 38, 42, 43, 45, 66, 74, 78, 94, 108, 111, 139, 140). In general, the principal aims of these studies have been to evaluate plaque and calculus removal with different types of root cleaning instruments and to examine the degree of tissue damage and/or change caused by the treatment. Neither hand nor ultrasonic cleaning had been found to totally remove plaque and calculus (31, 66, 108). In addition, no significant difference between the types of instruments has been noted in their efficiency in removing soft and hard deposits. It has also been reported that hand instruments remove substantially more tooth surface and that the topography of roots after the use of ultrasonics is less flat than that produced by hand scaling (108). Significant differences in tooth topography have been noted with respect to the type and/or sharpness of the instrument and the number of strokes used (42).

With sharpened ultrasonic tips the root surface has been found to be smoother than with dull ultrasonic tips, which produce a rubbed surface. Sharp curettes produce greater tissue alteration than dull ones (42). An index for SEM evaluation of root roughness has been proposed and new cleaning instruments, such as rotating diamond points, have been described as producing the most complete calculus removal (78, 94). There is evidence that ultrasonics are superior for the treatment of incisor teeth but have no particular advantage over hand instruments for the treatment of molars. More recently, a new ultrasonic device has been tested, but no difference from the degree of smoothness obtainable by the traditional ultrasonic dental unit was found (140).

A new attachment of connective tissue to the treated root surface has been considered for a long time to be one of the goals of periodontal treatment. This seems to be not likely on diseased root surfaces, which inhibit the "in vitro" attachment of fibroblasts, probably as a result of endotoxin adsorption (2, 3). Recent human studies have shown that root planing can remove the cytotoxic material that has adhered to or permeated the root surface to a level comparable to that found in healthy uninvolved teeth (99), thus allowing new connective attachment to the root. Several SEM investigations have dealt with the so-called "biological preparation" of the diseased root surface in order to detect a chemical treatment able to expose the collagen matrix of cementum and to detoxify the diseased root surface (74, 111, 139). Topical application of citric acid has been studied in humans (45) and in monkeys (111), on healthy and diseased root surfaces, untreated and root-planed. A three minute application of citric acid at pH 1 on root-planed teeth has been found to be able to remove the superficial smear layer that is produced during instrumental cleaning and to expose the orifices of dentinal tubules and intertubular zones with a fibrillar, mat-like morphology. Elastase and hyaluronidase, after the etching with citric acid seemed to result in even more effective exposure of the collagen matrix of the cementum (37). The effects on root morphology of topically applied EDTA, sodium hypochlorite, sodium deoxycholate, and Cohn's fraction IV have also been studied (74). Pretreatment of root-planed surface with fibronectin has been suggested to greatly enhance fibroblast attachment (43). However, the value of the creation of new attachment between connective tissue and the root surface is still controversial. In fact, epithelial attachments do not represent a "locus minoris resistentiae" (90), and root resorption can follow a new connective attachment (68).

To our knowledge, all the research conducted with the SEM in the field of root instrumental cleaning has been performed by studying the root topography only after cleaning without any types of information about the amount of deposit present on the root surface and its morphology prior to the instrumental manipulation. In our opinion, it is very difficult to evaluate the morphological changes which occur in an instrument treated root of a tooth that probably has had periodontal disease and instrumental cleaning prior to the experiment. A more reliable evaluation of root topography would be done by comparing the same area of the root before and after. An investigation has recently been performed to compare the morphology of the mineralizing front of cementum after the use of an ultrasonic device and hand instruments, using a technique that allowed identification of the original root morphology prior to cleaning and the comparison of the same root

area before and after the planing treatment (31) (Figs. 19 - 22). To achieve this goal, a replication technique was combined with SEM. Thirtyfour monoradicular periodontally involved teeth, rendered anorganic by 7% NaOCl solution, were divided into an ultrasonic instrument group (U.I.), a hand instrument group (H.I.), and a control group ,and then studied with the replica technique before and after treatment. The two replicas obtained from the same sample (before and after treatment) were mounted on a stub, sputter-coated, and examined by the SEM. The microscopist studied the same area of the two replicas at three different levels of magnification in order to assess the amount of calculus removed and the degree of tissue damage for the different instrumentation techniques.

The main results of the morphological analysis indicate that U.I. and H.I. exhibit similar levels of efficiency for both calculus removal and root planing. Complete root cleaning is not achievable with either curettes or ultrasonics, and curettes cause greater tissue damage. Since the results obtainable with ultrasonic and hand instruments are similar, it is rational from a cost-benefit point of view to suggest that an ultrasonic device is preferable for root treatment.

SEM : Other Contributions To Periodontal Research

Despite the great prevalence of periodontal disease, many aspects of the basic structure of the attachment tissue of the teeth remain unclear. The arrangement of collagen fibers in the periodontal ligament has vexed dental histologists for many years, especially about how the principal fiber bundles pass from tooth to bone.

It has been suggested that there is an intermediate plexus between fibers coming from the bone and those coming form the root surface (120, 121). SEM research, mainly by Sloan (123 - 125) has greatly contributed to a better understanding of the morphological organization of periodontal ligament in human (124, 130) and other mammals (123, 125). The periodontal ligament is organized in fibrous bands 100-150 μm width, which run longitudinally. The frequent branching and anastomosing and the course of these bundles makes it impossible to trace an intact network of bundles across the periodontal space. An intermediate plexus is probably an artefact of the methods of preparation (125). A vascular network running around the periodontal fibers has been studied in detail by the injection replica SEM method (59). Vascular and cellular changes in rat periodontal membrane after orthodontic treatment have been observed (81).

The epithelial-connective tissue interface of oral mucosa has been investigated in bioptic and autoptic human specimens (69, 103). Three regions with different characteristics of the epithelial-connective tissue have been identified : floor of the mouth, lip and cheek, gingiva and hard palate. Additional SEM research has been conducted on oral mucosa to describe the morphology of stratum corneum and to analyze the features of oral mucosa in different areas (35, 36, 71, 93). It has been deduced that the microplicae of superficial epithelial cells are more characteristic of non-keratinized cells in the oral cavity, while keratinized tissue more frequently is pitted or honeycomb in appearance, as is hard palate and the attached gingiva (71, 93). Futhermore, specificity of microbial distribution related to epithelial keratinization in Baboon tongue has been obtained with SEM (5). The number of bacteria inhabiting a mucosal surface seems to be related to the degree of keratinization.

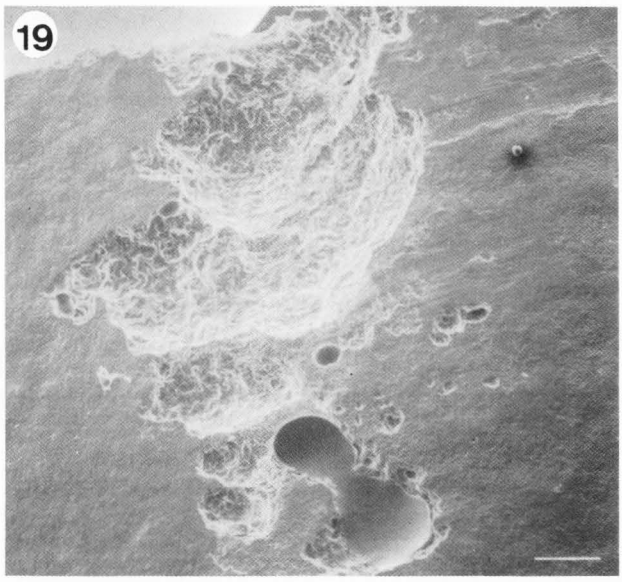
Information about the morphology of several of the major suspected periodontopathogens, such as Fusobacterium (21), Actinobacillus (Haemophilus) actinomycetemcomitans (57), Bacteroides (70) and Capnocytophaga (110), has been obtained by SEM. In order to differentiate between bacterial adhesiveness and invasiveness in cell culture monolayers, a method involving SEM has been proposed (23).

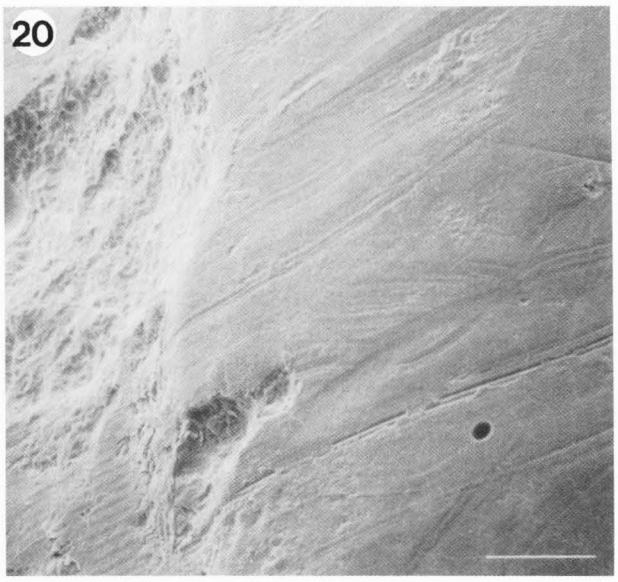
Spirochetes are now considered as possible periodontopathogens (for review see ref. 86). Patterns of "in vitro" interaction between Treponema denticola, human neutrophils and epithelial cells have been seen by SEM (101, 102). Treponema attach to epithelial cells preferably by their ends, whereas epithelial cells show no preferred site of Treponema attachment (101). Treponema was phagocytized by neutrophils in two major modes. The bacteria were either trapped by direct extension of the cytoplasmic membrane, forming a tight grip around them, or the bacteria sank directly into wide depressions that developed on the neutrophils surfaces (102). Other investigations in the microbiological field have dealt with the modality of plaque colonization on several materials such as glass (58, 138), hydroxyapatite (77), vestopal (8). These studies have shown that the specificity of plaque colonization depends on the substratum considered. Plaque colonization is also altered by chemical substances, i.e., chlorhexidine digluconate (141).

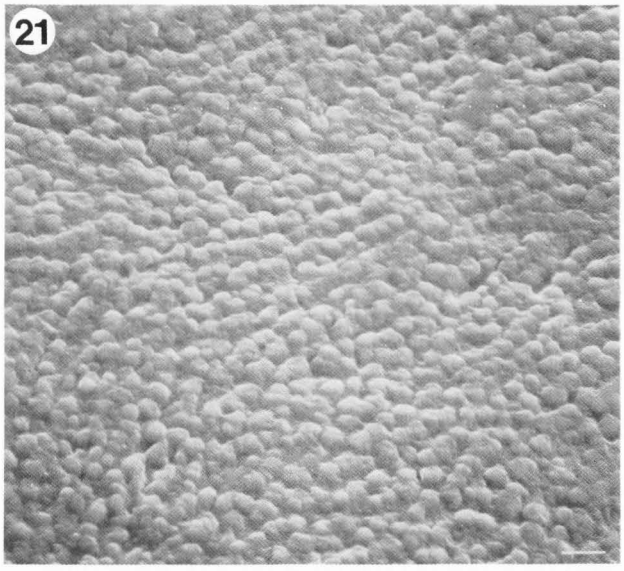
Concluding Remarks

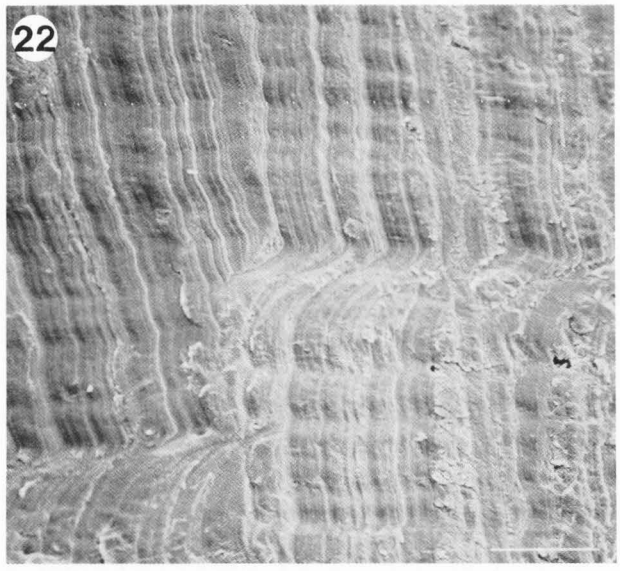
It is evident from the review of the literature that SEM has been widely and constructively used in periodontal research and that an improvement of our knowledge has been provided by this instrument. However, it is also clear that only a few groups have routinely utilized this type of microscope and that more frequently, SEM has been only occasionally used by researchers who, as a rule, work with other tools.

In our opinion the main reasons for this situation are either the expense of setting-up SEM laboratory or the lack of information that probably exists in the scientific community that works in periodontal research about the less common ways of using SEM. SEM has been used only in the traditional secondary mode, whereas other interesting methods, such as cathodoluminescence (20), and the back-scattered mode (18), have been so far neglected. These techniques could provide new information, i.e., regarding the distribution of dental plaque, stained in vivo with a disclosing solution, and the pattern of mineralization of alveolar bone and dental cementum in periodontal health and disease. Immunolabelling SEM techniques applied to periodontally involved teeth will represent a formidable tool to clarify the exact spatial location of specific types of bacteria. Finally, the problem of bacterial invasion into the gingival pocket wall could also be better understood with immunolabelling techniques and back-scattered emission microscopy on gingival sections.









Figs. 19 - 22. Replica technique. The same root areas before and after ultrasonic cleaning (**Figs. 19, 20**) and after hand cleaning (**Figs 21, 22**). **Bars** = 10 μm (on each figure)

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Discussion With Reviewers

J. Garnick: In the studies of morphology of root surfaces after instrumentation and with the use of the "anorganic" method of Jones, why study a non-diseased surface of the root where instrumentation will not be used in clinical treatment?

Authors: In our research on the effects of periodontal instrumentation on root morphology we have studied only periodontal involved teeth.

However, although we did not describe it in this paper, non-diseased root surfaces, prepared anorganically can provide useful information about the prevalence and the distribution of structures which might interfere with root treatment, such as cementicles, lateral formina, resorption areas.

J.Garnick: In the comparison of hand and ultrasonic instrumentation, can the use of extracted teeth determine the best type of instrument to be used in the mouth where the gingiva and tooth position may be a problem?

Authors: We agree with Dr. Garnick that an "in vitro" experiment aimed to study the effect of a clinical treatment has some limitations.

For analysis of root morphology after instrumentation, it could be claimed that the cleaning procedure is more easily done in the hands of the investigators than in the hands of the clinicians. This is basically true.

However, when different cleaning techniques are studied "in vitro" under the same experimental conditions and different results are constantly obtained, we can suppose that the same differences will hold under "in vivo" conditions.

Moreover, we were unable to use an "in vivo" model to compare the same area of the root before and after treatment, although such an experiment should be possible. In fact, we could use an "in vivo" replica technique to study areas of exposed cementum, such as recession, before and after root instrumentation. Unfortunately, this procedure is time consuming for the patient and the root surface below the gingival margin cannot be replicated by the impression material.

S.H. Ashrafi: Could SEM be used to identify the five different types of periodontal diseases?

Authors: During recent years we have studied the distribution and the composition of the microorganisms that inhabited the roots of more than 200 teeth extracted from patients with prepuberal periodontitis, juvenile periodontitis, rapidly progressing periodontitis and chronic adult periodontitis. We were unable to demonstrate morphological differences of the plaque which could identify the various morphological profiles in different periodontal conditions.

In our experience, it does not seem to be possible to identify a particular periodontal status by looking at the morphology of the plaque that covers the root by SEM.

S.H. Ashrafi: Do you think the cracks in the cementum are due to periodontal diseases or preparation artifacts? Why do you see more cracks in periodontal diseased cementum than in normal care?

Authors: Cracks on the cementum surface are con-

sidered an artefact due to differences in shrinkage during dehydration between the mineralized and the unmineralized components of the cementum. We noted that the greatest amount of damage to the cementum occurs during the coating process "in vacuo". This artefact could be avoided by using the replica technique.

We have not noted more cracks in periodontal diseased cementum. However, this has been reported by other authors and could be due to the exposure of cementum to the oral environment.

S.H. Ashrafi: How much useful information would an SEM study provide about the cuticular attachment of calculus matrix to tooth surfaces?
Authors: SEM could be usefully employed for the study of the interface between calculus and tooth if ultraflat sections are studied and backscattered electron images are used. The pattern of calculus mineralization and the relationship between cuticular attachment and tooth could also be studied with this technique.

R. Saglie: How would you recognize various types of phagocytic cells within gingiva by using SEM?
Authors: The only type of phagocytic cell that we are able to recognize by SEM in secondary mode is the neutrophil.

The identification of this cell is based on dimensions and typical surface morphology, characterized by lamellipodial projections.

A more detailed method of identification of neutrophils has been recently proposed. It uses backscattered electron imaging and colloidal gold as marker (de Harven, E., Soligo, D. 1986: Scanning electron microscopy of cell surface antigens labelled with colloid gold, The Am. Journal of Anatomy 175: 277-287).