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SCANNING ELECTRON MICROSCOPY OF BLACK STAIN ON HUMAN PERMANENT TEETH

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Abstract

Black stain may develop on the coronal surfaces of human teeth, and this type of stain is common in the Hong Kong Chinese population. The present study was undertaken to ascertain if the deposit conforms in composition to the black stain found elsewhere, and to describe its ultrastructure using the scanning electron microscope. Gram-stained smears were made from black stain on the teeth of 11 adult Hong Kong Chinese and studied microscopically. From another 15 persons extracted teeth exhibiting black stain were obtained and fixed. Two ground sections were made from each tooth, one was stained with toluidine blue, the other was dried and prepared for scanning electron microscopy. The gram-stained smears demonstrated predominantly gram-positive filamentous microorganisms with an admixture of gram-positive cocci and rods. The ground sections revealed a deposit on the outer surface of the enamel, which was clearly divided into two distinctly different layers: an inner yellow opaque layer, and an outer layer of microorganisms. Scanning electron microscopy demonstrated that the deposit consisted entirely of microorganisms, and that in portions close to the enamel they were often obscured by a substance indicative of calcification. Thus the black stain found on the teeth of Hong Kong Chinese is similar in composition and structure to that reported to occur in other populations. The black stain is a special type of dental plaque characterized by its simple flora and its tendency to calcify.

KEY WORDS: Black stain, dental plaque, dental calculus, extrinsic stain, permanent teeth, bacteriology, filamentous bacteria, cocci, rods, ultrastructure.

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Introduction

Various types of deposit may occur on the crowns of human teeth. One of these has been designated brown or black stain or plaque (Bibby, 1931; Pedersen, 1946; Leung, 1950; von Gülzow, 1963). Bibby (1931) described the stain as a brown line on the gingival third of the crowns of the teeth. He noted that in most cases the stain is separated from the gingival margin by a narrow unstained zone of enamel, although it was sometimes so advanced that it covered the entire gingival third of the crown. He also observed the stained deposit in the pits and fissures of the occlusal surfaces. The colour of the deposit varies between all shades of brown to almost black.

Structural studies of black stain have revealed it to consist of microorganisms adhering to the teeth, and consequently it may be classified as a special type of dental plaque, in which about 90% of the bacteria are gram-positive rods, most of them identified as actinomycetes (Slots, 1974). Like other types of dental plaque, the deposit may calcify to form dental calculus (Bibby, 1931; Theilade et al., 1973).

An interesting feature of the black stain is that it is often associated with a low caries frequency (Bibby, 1931; Pedersen, 1946; Shourie, 1947; Sutcliffe, 1967). In the Hong Kong Chinese population black stain is commonly present on the permanent teeth. The caries prevalence in Hong Kong is low (Lind et al., 1987). It was therefore decided to examine the black stain found on the permanent teeth of the local population to ascertain if the stain conforms in composition to that previously reported, and to describe further details of this black stain using the scanning electron microscope.

Materials and Methods

From 11 patients attending the Department of Periodontology and Public Health for periodontal treatment and displaying the typical brown to black stain on their buccal or lingual surfaces of the

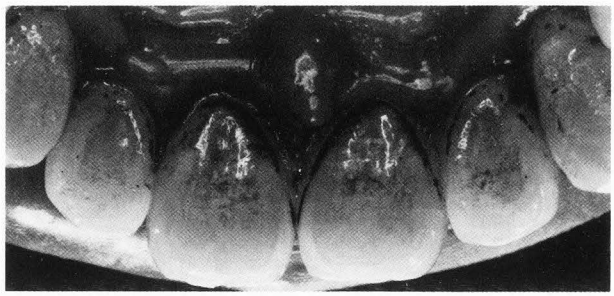

Fig. 1. Maxillary anterior teeth seen from the palatal aspect. Black stain is apparent as a distinct line parallel to the gingival margin, as a more diffuse deposit on the concave palatal surfaces of the teeth.

Fig. 2. Maxillary premolar and molar teeth with black stain on the palatal surfaces and in the fissures of the occlusal surfaces.

crowns of several teeth (Figs. 1 & 2), a sample of the stain was obtained by scraping the enamel surface with a curette. The adherent material was then suspended in a drop of water on a microscope slide, heat-fixed and subjected to the Gram-Twort staining method for microorganisms (Twort, 1924). These gram-stained smears were examined by transmitted light microscopy using oil immersion.

Five freshly extracted permanent teeth were obtained from different adult patients attending the Department of Oral Surgery and Oral Medicine of the Prince Philip Dental Hospital. The teeth were transferred immediately to a fixative containing a combination of paraformaldehyde and glutaraldehyde with 0.5 mg $CaCl_2$/ml (Karnovsky, 1965). After fixation for 18 h at 4°C, the specimens were rinsed in 0.1 M cacodylate buffer (pH 7.4) for 20 min. After the teeth were rinsed, two axiobuccolingual ground sections approximately 200 µm thick were prepared from each tooth using a home made sectioning machine equipped with a diamond-impregnated disc with a revolution speed of 2900 rpm (Mok and Fearnhead, 1985). One of the sections from each tooth was dehydrated and embedded in epoxy resin to support the section during subsequent polishing to a thickness of about 50 µm. This section was finally stained with toluidine blue and mounted on a microscopic slide for examination by transmitted light microscopy.

The other ground section from each tooth was postfixed subsequently in 2% osmium tetroxide in the same buffer after which it was again rinsed in the buffer, dehydrated in ascending concentrations of ethanol and subjected to critical point drying using freon. These sections were subsequently mounted on stubs on which they were sputter-coated with gold. The specimens were finally examined in a JEOL JXA-840 Scanning Electron Microscope (JEOL Ltd., 1418 Nakagami, Akishima, Tokyo 196, Japan) at 15-20 kV.

Another 10 teeth with black stain from different adult patients were obtained from the Prince Philip Dental Hospital Tooth Collection comprising extracted teeth stored in neutral buffered formalin (Jablonski et al., 1986). They were prepared in the same way as the freshly extracted teeth except that they were not further fixed nor subjected to critical point drying, so as to yield one ground section for light and another for scanning electron microscopy (SEM).

Results

Examination of the gram-stained smears revealed an unevenly distributed material consisting of bacteria between clumps of an opaque nature (Fig. 3). The microorganisms were predominantly long gram-positive filaments and gram-positive rods, particularly those in close association to

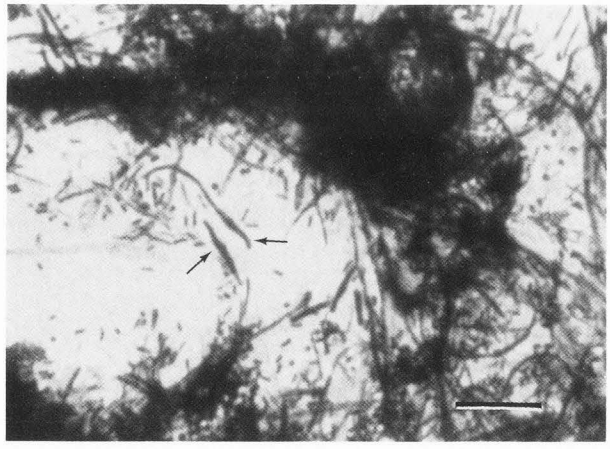

Fig. 3. Gram-stained smear of black stain. Long gram-positive filamentous organisms and gram-positive rods predominate. Some filaments have a thickened end (arrows). Bar = 10 µm.

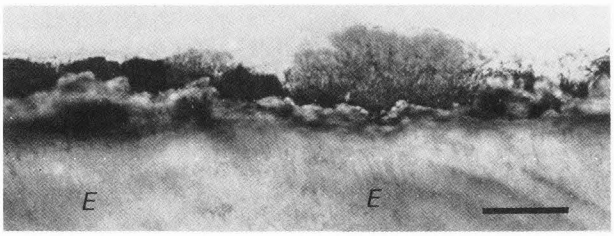

Fig. 4. Ground section of tooth with black stain. The enamel (E) is covered with deposits composed of two distinct layers: close to the enamel a yellow to brown layer and an outer bacterial layer taking up the toluidine blue stain. Bar = 100 μm.

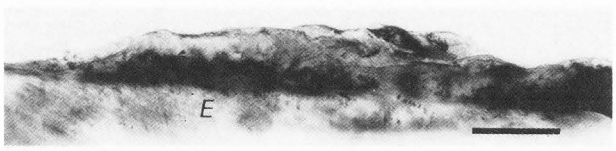

Fig. 5. Ground section of tooth with black stain. The enamel (E) is covered with a yellow to brown deposit with apparent layering. The outer bacterial layer is absent. Bar = 125 μm.

the clumps. Many of the long gram-positive filaments were segmented, consisting of short rods. Frequently the segmented organisms appeared to be surrounded by a sheath holding the individual segments together. Some of the gram-positive filamentous organisms were thickened at the end. Other filaments were studded with gram-positive cocci giving rise to corncob-like formations. Some areas of the preparation exhibited a more diverse flora including gram-negative cocci and rods conforming to the description of mature supragingival dental plaque. Epithelial cells were occasionally encountered.

Examination by light microscopy of the ground sections revealed a deposit of the outer surface of the enamel (Figs. 4 & 5). The deposit was clearly divided into two discrete layers, an inner yellow to brown layer, which did not stain with toluidine blue, and an outer layer of stained micro-organisms (Fig. 4). While each layer was occasionally absent, the inner opaque layer was most frequently present in the central portions of the deposits, while the stained bacterial layer was most prevalent in the periphery of the deposit, and often absent from the most protruding parts of the accretion (Fig. 5). The bacterial layer comprised mainly filamentous microorganisms deposited perpendicular to the enamel surface, thus exhibiting the typical appearance of dental plaque.

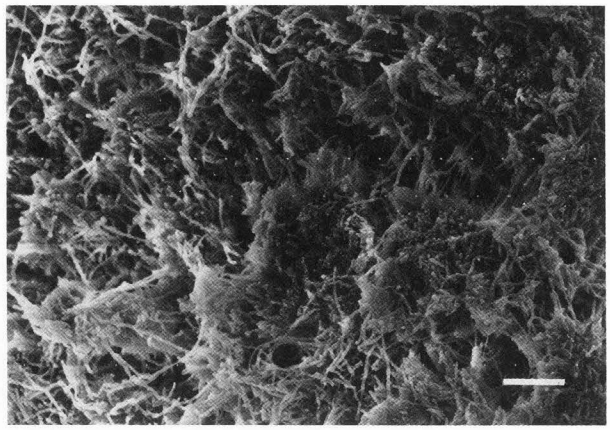

Fig. 6. Scanning electron micrograph of the outer surface of black stain. A predominance of long filamentous microorganisms is seen with cocci and rods between them. Bar = 10 μm.

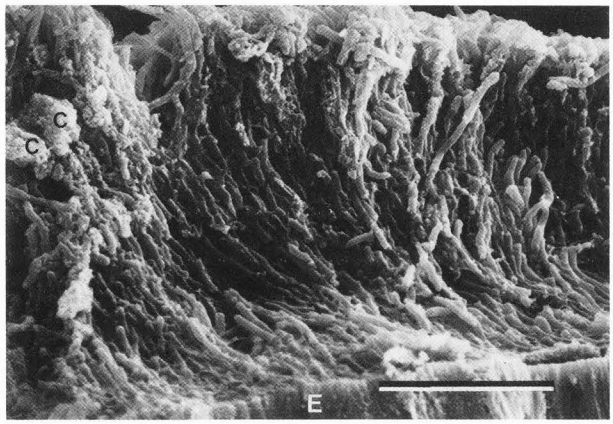

Fig. 7. Scanning electron micrograph of the cut surface of the ground section revealing the internal structure of black stain. Long filamentous microorganisms are deposited on the enamel (E) and extend to the outer surface of black stain. Some rods are present as are some filaments covered by cocci giving rise to corncob-like formations (C). Bar = 100 μm.

Further details of the surface structure were obtained from the SEM examination of the edge of the ground sections. In most areas of the samples the surface clearly demonstrated the bacterial nature of the deposit (Fig. 6). Long filamentous organisms predominated, between which coccoid bacteria were frequently seen. In some areas the bacteria were less discernible. They appeared to be covered by an amorphous

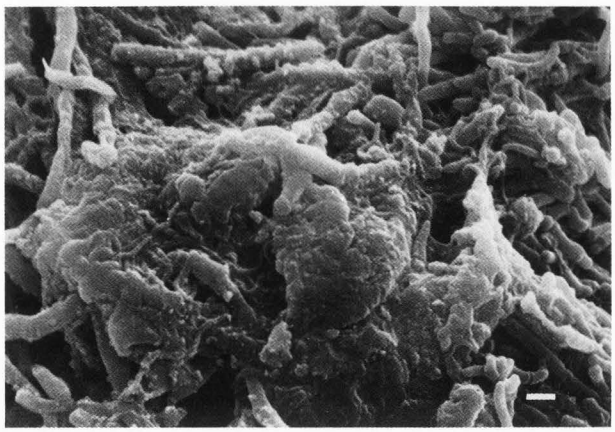

Fig. 8. Scanning electron micrograph of the outer surface of black stain. The micro-organisms are more or less obscured by a cementing substance. Bar = 1 μm.

material, most likely consisting of salivary precipitates. The internal structure of the deposit was studied along the cut surfaces of the ground sections of the teeth (Fig. 7). Where the deposit was comparatively thin, its bacterial composition could be ascertained in its full width, exhibiting a predominance of long filamentous microorganism oriented more or less perpendicular to the tooth surface. In some parts of the deposit, the morphology of the microorganisms was obscured by a cementing substance (Fig. 8). Scanning electron microscopy did not reveal any obvious difference between the morphology of the deposits that had been formalin-fixed and air-dried and those fixed in glutaraldehyde-paraformaldehyde and subsequently critical point-dried.

Discussion

Examination of the black stain frequently found on the coronal surfaces of the teeth in the Hong Kong population revealed that the structure of the deposit conforms to earlier descriptions of such deposits (Bibby, 1931; Theilade et al., 1973).

Furthermore, examination of the gram-stained smears revealed that the predominant organisms of the deposit were gram-positive filamentous microorganisms, an observation in line with the descriptions previously reported concerning such stains. Thus Bibby (1931) isolated seven thread-forming organisms of which two aerobic forms, both highly pleomorphic, grew readily in sub-culture as branching threads, sometimes with sheath formation and bacterial fragmentation, or as bacilli. In the present study the long gram-positive filaments also appeared to possess an external sheath and to exhibit fragmentation into short rods. In sections of teeth coated with a surface film, Bibby and van Huysen (1933) also noted that the film contained "a layer of gram-positive thread-forming bacteria arranged like a picket fence with the elements at right angles to the surface". The predominance in black stain of gram-positive organisms was similarly noted by Theilade et al. (1973) in a transmission electron microscopic examination of thin sections, and in a newer cultural study of black stain on primary teeth it was found that gram-positive rods averaged 90% of the cultivable organisms, most of them identified as actinomycetes (Slots, 1974).

In addition to the predominating gram-positive filaments and rods, a smaller number of other types of bacteria, many of which were gram-negative, were encountered in the present investigation of gram-stained smears from the black stained deposits. These bacteria are typical of mature dentogingival plaque (Theilade et al., 1966) and not of black stain. An admixture of such organisms was also noted in thin sections examined by transmission electron microscopy (TEM), particularly at the outer surface of the deposit (Theilade et al., 1973).

The ground sections of the teeth with black stain showed that the deposit comprised two distinct layers, the outer bacterial layer and an inner opaque, yellow to brown material resembling dental calculus adjacent to the enamel. Dental calculus has been shown to consist of calcified dental plaque (Gonzales and Sognnaes, 1960; Theilade, 1960; Zander et al., 1960). The observation of portions of the deposit, in which the microorganisms was obscured by a cementing substance, is most likely a result of calcification of the bacteria, in accordance with previous investigations of black stain. Thus Bibby (1931) reported that the deposits contained small quantities of inorganic substances, possibly in the form of calcium, magnesium, phosphates and carbonates, and Reid and Beeley (1976) found that the calcium and phosphate content of black stain was higher in black stain than in gingival debris from individuals with no stain. TEM revealed that calcification of the deeper layer of the bacterial aggregates in black stain occurred in a pattern indistinguishable from that of dental calculus (Theilade et al., 1973). It can thus be concluded that the black stain found commonly on the permanent teeth of the Hong Kong Chinese does not differ basically in structure and bacterial composition from that of similar stains in other populations. The black stain is a special type of dental plaque characterized by its rather simple flora and its tendency to calcify. This explains why once formed the stain cannot be readily removed without the professional assistance of dental personnel.

The prevalence of black stain varies considerably (Pedersen, 1946; Shourie, 1947;

Leung, 1950; von Gülzow, 1963; James, 1965; Sutcliffe, 1967; Gerdin, 1970; Samuelson et al., 1971). Bibby (1931) noted that the black plaque was more obvious in less cleaned mouths and was of the opinion that its development could be prevented by assiduous brushing. The common occurrence of black stain in the Hong Kong Chinese may also be related to their oral hygiene practices, as a recent survey indicated a great need for improved oral hygiene (Lind et al., 1987).

Aside from its unsightly appearance, black stain does not seem to have any direct detrimental effect on the oral tissues. This does not preclude that it could have an indirect effect in promoting the establishment of the typical dentogingival plaque along the gingival margin resulting in gingival inflammation. Contamination of the typical black stain flora with the complex mixture of microorganisms characteristic of dento-gingival plaque may support such a contention. If so, the logical consequence would be to remove the stain from the teeth and prevent its recurrence through improved oral hygiene measures.

Conclusions

The extrinsic black stain commonly found on the permanent teeth of the Hong Kong Chinese conforms in structure and bacterial composition to that found in other populations. It is a special type of dental plaque characterized by a simple bacterial flora predominated by filamentous micro-organisms, and has a tendency to calcify, making its removal difficult without the professional assistance of dental personnel.

Acknowledgements

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Discussion with Reviewers

H.N. Newman: Do you distinguish between black/brown stain of the Bibby-type and other dark extrinsic stains?
Authors: All the stains examined conformed to the description offered by Bibby. However, other types of black/brown stains have been reported, as for instance the dark extrinsic stain of betel nut chewers (Reichart et al., 1985) and that occurring after prolonged use of chlorhexidine gluconate mouthwashes (Eriksen and Gjermo, 1973), but such stains were not encountered in the present material.

H.N. Newman: You describe the black stain plaque as simple, because it consists of cocci and filaments. Isn't this very similar to conventional early supragingival plaque?
Authors: Early supragingival plaque also has a simple, gram-positive flora, but different from that of black stain (Theilade et al., 1966, Theilade et al., 1982). It is dominated by streptococci and minor proportions of Actinomyces, while the black stain is predominantly Actinomyces and other gram-positive rods and filaments. The Actinomyces are pleomorphic and may appear as coccoid cells, rods, or filaments depending on the growth conditions.

H.N. Newman: Have you any evidence as to the factors responsible for the color of the deposit?
Reviewer IV: What is the origin of the black stain? Is it due to special types of micro-organisms? Or is it due to a chemical stain related to special oral habits?
R.W. Fearnhead: What is the nature of the black pigmentations? Could it in anyway be related to Indigo? As you know Indigo is a natural dye produced from indican, a glucoside, by fermentation. Thus given the appropriate ingredients in the diet and microorganisms capable of providing the fermentation, the oral environment might well provide a suitable niche for the production of small amounts of this dye.
Authors: This investigation was not designed to reveal why the deposit is black. However, it has been proposed that the color is the result of bacterial metabolism, Bacteroides melaninogenicus being the suspect, because it has the oral cavity as its normal habitat and may produce black colonies in vitro. This organism is capable of producing a hemin-like compound, when grown on blood agar. However, on partially defined medium containing excess ferrous ions, it produces no hemin, but ferrous sulphide. Other hydrogen sulphide forming bacteria also produce this compound indicating that bacterially formed hydrogen sulphide is converted to the black ferrous sulphide (Reid, Beeley and MacFarlane, 1976). Bacteroides melaninogenicus is reported to appear in greater numbers on the teeth of children with black stain, and scrapings of the black stain contains more iron than scrapings from teeth with no black stain (Reid, MacDonald and Beeley, 1974). On the other hand, in another study, in which extensive characterization of the bacterial flora of black stain of children was carried out, Bacteroides melaninogenicus only constituted less than 1% of the cultivable organisms on blood agar plates. It was therefore considered unlikely that this organism was related to the color of the stain and other hydrogen sulphide producing bacteria were not predominant either (Slots, 1974). Thus, the factors responsible for the color of the stain remain uncertain.

Whether Indigo may contribute to the stain is not known, but an experiment could be set up to test this hypothesis.

H.N. Newman: You say it could have an indirect effect in promoting gingivitis. What differences, if any, have you or others noted as regards relationship between black stain and conventional plaque and gingivitis?
Authors: If dental plaque is allowed to accumulate at the gingival margin, inflammation of the gingiva invariably occurs (Theilade et al., 1966). The relation between black stain and gingivitis has not been investigated. Black stain or other coronal plaque which is situtated without direct contact with the gingival tissues is unlikely to have a detrimental effect on the gingiva. If the black stain acts as a retention site for bacterial accumulation at the gingival margin, gingivitis is unavoidable.

J. Crawford: Figure 1 shows that the distribution of the stain in this case is parallel to the gingival margin but separated from it by an unstained zone. Would the investigators please speculate on the role of crevicular fluid in this distribution? Specifically, in areas of gingival health, is this unstained area absent?
Authors: The oral ecosystem comprises several distinct habitats each supporting the growth of a characteristic microbial community adapted to the local environment (Theilade and Theilade, 1985). Even subclinical inflammation of the gingival margin will result in the formation of gingival exudate, which will support the growth of a flora different from coronal plaque situated further away, where the exudate is absent or very diluted. It may be that the gingival exudate inhibits the organisms reponsible for the black pigment either directly, or indirectly by supporting preferentially the gingival flora at the expense of the coronal flora further away from the gingival margin. If this is so, the unstained zone should be absent in gingival health. This hypothesis is still untested.

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