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ISOLATION OF PEPTOCOCCI AND PEPTOSTREPTOCOCCI IN DEVELOPING HUMAN DENTAL PLAQUE BY MAINTAINING CONTINUOUS ANAEROBIOSIS

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Plaque samples of 2, 5, 7 and 12 days were obtained under complete anaerobiosis with a special technique from the labial surfaces of the upper incisor teeth of six subjects. The samples were primarily assayed for their content of grampositive bacteria; actinomyces, eubacteria, lactobacilli, corynebacteria, nocardia, propionibacteria, peptococci, peptostreptococci, and streptococci. At day 2 streptococci, corynebacteria and nocardia predominated, but after 5 days also anaerobic gram-positive cocci and rods were found. 15 strains of these cultivable gram-positive anaerobic cocci were investigated further with regard to morphology, biochemical reactions and fermentation products. Seven of these organisms resembled *Peptostreptococcus intermedius* and four of these strains were probably *Peptostreptococcus anaerobius.* One appeared to belong to *Peptococcus magnus* and the remaining strains could be considered as *Peptococcus variabilis.*

Anaerobic bacteria are now recognized as a part of the human oral flora (*Rosebury*, 1962). It is known that most of these anaerobic bacteria cannot be cultured with conventional anaerobic techniques (*McMinn & Crawford,* 1970). Thus the prereduced anaerobically sterilized culture media are capable of recovering more than twice the number of anaerobic bacteria from clinical specimens that could be recovered by conventionally used media.

Although gram-positive cocci are the most common bacteria in the oral cavity and appear to play an important role in the pathogenesis of oral diseases, very little is known about anaerobic gram-positive cocci. The aim of this investigation was to study anaerobic gram-positive cocci in developing human dental plaque.

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MATERIALS AND METHODS

Six subjects (5 men and 1 woman) between 25—31 years of age participated in this study. At the beginning of the experimental period all teeth in the upper and lower jaw were carefully pumiced to remove plaque and calculus. The subjects were then instructed to avoid artificial oral hygiene procedures. No changes in their diet were made.

Preparation of media and diluent. Preparation of prereduced anaerobically sterilized media and diluent was made according to the method described by *Holdeman & Moore* (1972). The basal medium contained peptone (Difco) 2.0 g, yeast extract (Difco) 1.0 g, resazurin solution 0.4 ml, salt solution 4.0 ml, cysteine-HCl . H_2O 0.05 g and distilled water 100 ml. The salt solution consisted of $CaCl_2$ 0.2 g, $MgSO_4$ 0.2 g, K_2HPO_4 1.0 g, KH_2PO_4 1.0 g, $NaHCO_3$ 10.0 g, NaCl 2.0 g, distilled water 1000 ml. All ingredients except cysteine-HCl were mixed in an Erlenmeyer bottle. The medium was boiled in 20 minutes until the medium changed from pink to yellowish. The bottle was then cooled in ice water under oxygen-free nitrogen. After cooling, the flask was removed from the ice-bath and cysteine-HCl was added. The medium was dispensed in tubes. The tubes were stopped with recommended black butyl rubber stoppers. The closed tubes were placed in a press and autoclaved for 15 minutes at 121°C. The following fermentation media were used; peptone-yeast extract basal medium with cellobiose, fructose, glucose, lactose, maltose, sucrose: Gelatin-medium for gelatin liquefaction. Chopped meat for indol production; Nitrate-medium for nitrate reduction and Brain Heart Infusion agar for catalase production. The diluent contained peptone, yeast extract, cysteine-HCl . H_2O, 0.01 M phosphate buffer (pH 7.0) and 0.001 % resazurin. The pH of the media and diluent were checked with a pH-meter pHM 51 (Radiometer, Copenhagen, Denmark).

Dental plaque samples. Dental plaque was collected from the facial surface of one anterior tooth in the upper jaw on days 2, 5, 7 and 12 from each one of the six subjects. The plaque material was obtained with a sterile dental scaler. Care was taken to avoid sampling material from the gingival crevices or the approximal spaces. Each plaque sample was immediately placed in a tube with reduced diluent and homogenized in a mixer (Whirl-mixer, Lab. line, USA) for 30 sec. under continuous flow of oxygen-free nitrogen. It was possible to execute this procedure without oxidation of the resazurin in the diluent. During the sample procedure a special mobile anaerobe unit was used (*Fulghum*, 1971). It consisted of a cylinder containing 3 % hydrogen in nitrogen with a metal valve (AGA, Stockholm, Sweden) supplying the gas.

The gas mixture was led from the cylinder via a copper tube to a catalyst (Deoxy, model D5/05, Engelhart, London, England) which removed oxygen from the gas mixture. The gas was then dispensed via a butyl rubber tube over the buccal surfaces of the teeth during collection of plaque.

Culture methology. The anaerobic culture system (7790—11111, Bellco Glass, New Jersey, USA), agar tube streaker (7790—33333, Bellco Glass) and agar tube spinner (7790—44444, Bellco Glass), developed by the Virginia Polytechnic Institute and State University, Anaerobe Laboratory, were used in this study. Oxygen-free gas used for preparation of media and for inoculations was obtained as described above. One-tenth ml of the sample and tenfold dilutions of the sample were incubated in roll tubes with prereduced agar medium under complete anaerobiosis. The roll tubes were examined with the aid of a microscope for careful differentiation and picking of bacterial colonies. Anaerobes that grew in prereduced media were always reinoculated into dextrose-serum broth to determine their ability to grow in that medium. An additional check was inoculation of organisms from prereduced media into aerobically incubated blood agar plates. All tubes and plates were incubated at 37°C for 4 days. Identification of anaerobic bacteria was based on morphology and biochemical reactions recommended by *Holdeman & Moore* (1972). Tests for gelatin liquefaction, nitrate reduction, indol production, catalase production and lowered pH in media containing cellobiose glucose, fructose, sucrose, lactose and maltose were carried out.

The reference strains used — *Peptostreptococcus intermedius* 7292 B, *Peptostreptococcus anaerobius* 4330, *Peptococcus magnus* 4287 and *Peptococcus variabilis* 5662 — were obtained from Professor L. Holdeman, Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

Analysis of acid and alcohol products. The culture supernatants of peptone-yeast extract glucose medium (PYG) were analyzed for volatile acids and alcohols by gas chromatographic analysis in a gas chromatograph model Varian 920 (Varian AB, Solna, Sweden). The column was filled with chromosorb G, the carrier gas (He_2) flow rate was 50 ml per min and the temperature was 110—130° C.

RESULTS

Proportions of aerobic and anaerobic gram-positive microorganisms in different ages of plaque samples. The relative proportions of aerobic and anaerobic gram-positive microorganisms in developing dental plaque varied

during the experimental period. The plaque at day 2 seems to be made up primarily of aerobic and facultative streptococci, corynebacteria and nocardia. With development of the plaque (day 5), a second phase of bacterial proliferation was observed. It was characterized by gram-positive rods and filamentous forms, but streptococci were still present in large numbers. At days 7—12 the predominant types of gram-positive microorganisms were strains belonging to streptococci, peptococci, peptostreptococci, lactobacilli, actinomyces, corynebacteria and propionibacteria.

Recovery of peptococci and peptostreptococci. The time required for peptococci and peptostreptococci to appear in prereduced media changed from 24 hours to 4 days. When the culture appeared to be more than one organism present, or when biochemical reactions were ambiguous, careful observations of prereduced agar slant cultures were made with a microscope. Colonies with good separation and different colonial morphology were subcultivated in prereduced media and on aerobically incubated blood agar plates. However, several attempts to separate colonies on prereduced agar slants were required before a good separation could be achieved. Detailed identification was only attempted for strains belonging to *Peptococcus* and *Peptostreptococcus*.

Characteristics of peptococci and peptostreptococci

Fifteen microorganisms of the isolated strains, primarily identified as strains belonging to peptococci and peptostreptococci, were selected for further characterization. All strains were gram-positive cocci growing singly, in pairs, tetrads or chains. They were non-motile and contained neither catalase nor oxidase. Further characteristics of the strains are given in the following text.

Group 1. Gram-positive spherical to ovoid cells 0.6—0.7 μm occurring in short or long chains. Acid from cellobiose, fructose, glucose, lactose, maltose and sucrose. Gelatin not liquefied. Indol not produced. Nitrate not reduced. No catalase activity. Lactate was the major product in peptone-yeast extract-glucose medium. This first group resembled *Peptostreptococcus intermedius* (seven strains).

Group 2. Gram-positive spherical cells 0.8 μm, occurring in chains. Acid from glucose and fructose. No acid from cellobiose, lactose, maltose and sucrose. Gelatin not liquefied. Indol not produced. Nitrate not reduced.

No catalase activity. Fermentation product mainly acetic acid, traces of isobutyric, butyric and isovaleric acids. This second group resembled *Peptostreptococcus anaerobius* (four strains).

Group 3. Gram-positive spherical cells 1.3—1.7 μm occurring singly, in pairs or tetrads. No acid from cellobiose, fructose, glucose, lactose, maltose and sucrose. Gelatin not liquefied. Indol not produced. Nitrate not reduced. No catalase activity. Fermentation product mainly acetic acid. This third group resembled *Peptococcus magnus* (one strain).

Group 4. Gram-positive spherical cells 0.6—1.0 μm occurring singly, in pairs or tetrads. Acid from fructose and glucose. No acid from cellobiose, lactose, maltose and sucrose. Indol not produced. Gelatin liquefied. Nitrate not reduced. No catalase activity. Fermentation product mainly acetic acid. This fourth group resembled *Peptococcus variabilis* (three strains).

DISCUSSION

During the initial phase of plaque formation deposition of salivary glycoproteins occurs on the surfaces of the tooth. The coating of these surfaces with glycoproteins prepares the surface of the tooth for bacterial colonization (*Nord, Linder, Wadström & Lindberg,* in press). Environmental conditions at this time are such that only the aerobic and facultative mircoorganisms can grow. Thus, the build-up of dental plaque is primarily due to aerobic growth and the increase in extracellular polysaccharide-producing microorganisms results in increased bacterial polysaccharides in the plaque (*Leach,* 1970). The proper environment for anaerobic growth will then occur. In this study it was also shown that the proportions of aerobic organisms declined with the progression of plaque, and the anaerobic bacteria increased in proportions as plaque grows. The shifts in the relative proportions of the microorganisms may be due to the changes in the oxidation-reduction potential occurring in developing plaque (*Kenney & Ash,* 1969).

When the anaerobic jar method is used, samples are exposed to air during a short period in which the bacteria are spread over the agar surface. This brief exposure to air is sufficient to kill many anaerobic microorganisms (*Loesche,* 1969), because many anaerobes are very sensitive to oxygen and are different from others that can survive short exposures to air and even grow in the presence of low concentrations of oxygen. The roll tube technique, however, permits significantly higher recoveries of anaerobic microorganisms

than the anaerobic jar method (*Moore*, 1966) and was found to be a simple procedure for obtaining an anaerobic environment suitable for the isolation of peptococci and peptostreptococci in dental plaque. *Gordon, Stutman & Loesche* (1971) also recently showed improved isolation of anaerobes from the gingival crevice of man by using the roll tube technique. The superiority of prereduced media used in this study for detection of anaerobic microorganisms can be attributed to the reduced state of medium components and to an oxidation-reduction potential of — 100 mV (*Moore, Cato & Holdeman*, 1969).

The most difficult problem of maintaining anaerobiosis occurred during the collection of plaque material. Organic peroxides may be formed during collection in an athmosphere containing oxygen and may kill anaerobic bacteria. This was avoided by direct inoculation of prereduced rubber-stopped tubes at the patient and by continuous flushing on the teeth with oxygen-free gas during collection.

It must be pointed out that many oral bacteria are facultative and grow well under anaerobic conditions as obligate anaerobes do. Therefore microorganisms recovered from roll tubes were subcultured aerobically and anaerobically to determine which were obligate anaerobes.

In *Bergey's Manual* (1957) anaerobic gram-positive cocci are placed in two genera on the basis of the arrangement of cells. Cocci that have cells occurring in pairs or in chains are placed in the genus *Peptostreptococcus* in the family *Lactobacillaceae* and those that occur singly, in pairs, tetrads or masses are located in the genus *Peptococcus*, family *Micrococcaceae*. *Prévot* (1966) did not place anaerobic gram-positive cocci in separate genera. He placed the species in the genera with the aerobic and facultative cocci that have similar morphological characteristics, for example *Streptococcus, Staphylococcus, Micrococcus*. However, differentiation of peptococci and peptostreptococci on the basis of morphological characteristics is sometimes not easy, because both genera contain species occurring in chains. *Rogosa* (1971) recently proposed a new family, *Peptococcacae*, in the order *Eubacteriales* including three genera of gram-positive anaerobic cocci — *Peptococcus, Peptostreptococcus* and *Ruminococcus*. Separation of peptococci and peptostreptococci on the basis of catalase production is also questionable. Members of *Micrococcacae*, including peptococci, are described to produce catalase, while members of *Streptococcus* including *Peptostreptococcus* are described as not producing catalase. However, some strains of peptococci do not produce catalase and this property is not well established as a property for anaerobic gram-positive cocci. In our study no strains were found to produce catalase.

In this work it was also found that most gram-positive anaerobic cocci in dental plaque were peptostreptococci. *Smith & Holdeman* (1968) also reported in a study of anaerobic gram-positive cocci isolated from man and animals that 70 per cent were »anaerobic streptococci», 15 per cent were »anaerobic staphylococci» and 15 per cent were »anaerobic diplococci». The gram-positive cocci are a complicated and troublesome group. There are a large number of microorganisms with apparently distinguishable characteristics that might be put into separate species. However, most of the anaerobic gram-positive cocci cannot be identified with certainty according to recommendations in *Bergey's Manual* (1957). The morphology of oral anaerobic gram-positive cocci is therefore not so clear as in the case of oral anaerobic gram-negative rods, and this first work is followed by further studies on oral anaerobic gram-positive cocci that will be reported soon.

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