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Streptococci and Actinomyces Inhibit Regrowth of *Streptococcus mutans* on Gnotobiotic Rat Molar Teeth after Chlorhexidine Varnish Treatment

Key Words

Actinomyces naeslundii
Chlorhexidine
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Interference
Streptococcus gordonii
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Streptococcus mutans
Streptococcus oralis
Streptococcus sanguis

Abstract

Clinical studies suggest that the long-term suppression of mutans streptococci on tooth surfaces after intensive chlorhexidine therapy is mainly due to bacterial interference. Other streptococci and also *Actinomyces naeslundii* are proposed to inhibit regrowth of mutans streptococci after suppression by the agent. We have tested this hypothesis in gnotobiotic rats associated with *Streptococcus mutans* alone, or associated with *S. mutans* and strains of *Streptococcus oralis*, *Streptococcus sanguis*, *Streptococcus gordonii*, *Streptococcus mitis* biovar I, and *A. naeslundii*. Left lower jaws in these rats were treated with concentrated chlorhexidine varnish, and the return of *S. mutans* on the treated jaws monitored. In mono-associated rats, *S. mutans* regained the level of the untreated right lower jaw in approximately 1 week. In contrast, *S. mutans* remained suppressed for several weeks in rats multi-associated with other streptococci and actinomyces strains. The suppression was more pronounced in the rats fed on basal diet with little free sugars than in rats fed on a sucrose-containing diet. Counts of other streptococci recovered quickly from the intensive chlorhexidine treatment, but *A. naeslundii* remained suppressed for at least 1 week. The findings demonstrate the crucial importance of the oral microflora in controlling regrowth of mutans streptococci after chemotherapy.

Various chemotherapeutical regimes have been used successfully to suppress mutans streptococci on the human dentition [Emilsson et al., 1976; Caufield and Gibbons, 1979; Malz et al., 1981; Zickert et al., 1982; Schaeken and de Haan, 1989; Ostela et al., 1990; Sandham et al., 1991]. We have earlier ascribed the long-term suppression of mutans streptococci after stop of chemotherapy to bacterial interference. Their growth after chemotherapy seemed to be inhibited

by other microorganisms in dental plaque, and particularly by other streptococci and actinomyces [Schaeken et al., 1989]. This hypothesis stems from observations that experimental colonization of sites of the dentition by mutans streptococci is often difficult, and spread of the organisms throughout the mouth is limited [Mikx et al., 1975; Svanberg and Loesche, 1977, 1978; van der Hoeven and Rogers, 1979]. Further support for microbiological control of *Strep-*

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Staphylococcus mutans populations in ecosystems comes from mixed-culture chemostat studies and artificial mouth models [McDermid et al., 1989; Perrons and Donoghue, 1990].

In the study described here we have attempted to confirm the bacterial interference hypothesis as follows. Regrowth of a *S. mutans* strain after chemotherapy was monitored in gnotobiotic rats mono-associated with the organism, as well as in gnotobiotic rats harbouring several known streptococcal and actinomyces strains in addition to *S. mutans*. The strains used were isolated from human fissure plaque shortly after treatment with chlorhexidine varnish [Schaeken et al., 1994]. The rats were treated with chlorhexidine varnish to eliminate *S. mutans* and to study its regrowth. The varnish treatment was limited to one lower quadrant, leaving *S. mutans* on other sites of the dentition and in saliva virtually undisturbed. Thus, a high colonization pressure of *S. mutans* on the treated surfaces was maintained.

Methods and Materials

Microorganisms and Inoculations

The strains used in this study were all isolated from human fissures during a previous experiment [Schaeken et al., 1994]. They were obtained from plaque samples collected shortly after the fissures had been treated with chlorhexidine varnish. The strains, speciated according to Kilian et al. [1989], were *Streptococcus oralis* Ny 592, *Streptococcus mitis* biovar I Ny 593, *Streptococcus sanguis* biovar I Ny 594, *Streptococcus gordonii* biovar II Ny 595, *Streptococcus mutans* Ny 591. Further *Actinomyces naeslundii* strains Ny 596 and Ny 597 were used. The strains were kept in skimmed milk at -90°C . For inoculation, the organisms were grown for 18 h in tryptone-yeast extract (Difco, Detroit, Mich., USA) broth containing 0.1% galactose. Galactose was used as an energy source because glucose may cause repression of glycosidase activities. The *S. mutans* culture was used directly to inoculate the rats. The cells of the other streptococci and actinomyces strains were centrifuged at 5,000 g and resuspended in 20% of the original volume of the same medium. The inoculum was prepared by mixing equal volumes of the concentrated cell suspensions. The rats were inoculated by giving them 100- μl amounts of the inoculum in the mouth using a syringe. In order to reduce variation, the inoculation of the whole group of rats was repeated twice. This inoculation procedure was repeated the next day. To guarantee its establishment, *S. mutans* was inoculated first on 2 successive days. The other strains were inoculated 1 week later, except in the group of rats that remained mono-associated with *S. mutans*.

Animals and Treatments

Six litters of 8 germ-free Wistar rats were equally distributed among two dietary groups. The rats were 28 ± 2 days old. They were kept in plastic isolators during the experiment. One group received the basal diet of the following composition: wheat flour, 59.2%; skimmed-milk powder, 32%; lyophilized baker's yeast, 7%; soya oil, 1%; vitamin mixture according to Gustafson's diet D7 [Luckey, 1963], 0.8%. The other group of rats was fed the same diet but with 5% sucrose substituted for wheat flour. At the start of the experiment all rats were

inoculated with *S. mutans* on 2 successive days, and 1 week later the multiple-associated rats were further inoculated with the above streptococcal and actinomyces strains. Two weeks after the last inoculation, the left lower jaws of the rats were treated with 40% chlorhexidine varnish [Schaeken and de Haan, 1989]. To this end, the rats were temporarily transferred to a laminar flow cabinet. They were anaesthetized and, after drying the dentition with air, the molar teeth in the left lower jaw were covered with chlorhexidine varnish using a syringe. One, three, and six weeks after varnish treatment, samples of 8 rats were taken from each group. The rats were killed in an atmosphere of CO_2 , and the molars in the left and right lower jaws were extracted, giving two samples per rat. The molars were ground in a mortar, ultrasonically dispersed in saline using a Kontes micro-ultrasonic cell disrupter (model 9110001, Kontes, Vineland, N.J., USA) as described before [Beckers and van der Hoeven, 1982]. Appropriate dilutions of the suspensions were plated onto blood agar, CNAC agar for enumeration of *A. naeslundii* [Ellen and Balcerzak-Raczkowski, 1975], and TSY20B agar for enumeration of *S. mutans* [Schaeken et al., 1986]. The plates were incubated in an atmosphere of 91% N_2 , 5% CO_2 , and 4% H_2 for 4 days at 37°C .

The data were $\log_{10}$ -transformed prior to statistical analyses. The data for *S. mutans*, *A. naeslundii*, and other streptococci were subjected to ANOVA. For each diet group and at each sampling occasion, the treatment effect (chlorhexidine vs. control) was evaluated using Student's *t* test.

Results

The numbers of *S. mutans* on the molar teeth in the chlorhexidine-treated and control lower quadrants 1, 3 and 6 weeks after varnish treatment are shown in figures 1 and 2. There is a marked difference in the regrowth of *S. mutans* on the chlorhexidine-treated jaws between the rats mono-associated with the organism (fig. 1) and the multiple-associated rats harbouring the streptococcal-actinomyces microflora (fig. 2). One week after chlorhexidine treatment the number of *S. mutans* in mono-associated rats had already returned to approximately 50% of its level on the untreated right lower jaws. In contrast, the suppression of *S. mutans* in multi-associated rats was much stronger and persisted for several weeks. Six weeks after treatment, the mean number of *S. mutans* on chlorhexidine-treated jaws in the basal diet group was still less than 1% of its number on the control site. The suppressive effects were more pronounced in the group of rats fed on the basal diet with little free sugars than in the rats fed on the sucrose diet. In contrast to *S. mutans*, levels of other streptococcal strains returned to the level of the control right lower jaw within 1 week after chlorhexidine treatment (data not shown). *A. naeslundii* strains were initially reduced by chlorhexidine treatment (fig. 3). Suppression was strongest in the group of rats on the basal diet; 6 weeks after chlorhexidine application, *A. naeslundii* on the treated jaws had reached control-site levels.

Fig. 1. *S. mutans* in two diet groups of mono-associated gnotobiotic rats 1, 3 and 6 weeks after chlorhexidine treatment. Each bar gives the numbers of *S. mutans* on the chlorhexidine varnish-treated left lower jaws (full) and the untreated right lower jaws that served as control (open). * $p < 0.05$; ** $p < 0.01$.

Fig. 2. *S. mutans* in two diet groups of gnotobiotic rats multi-associated with *S. mutans*, *S. oralis*, *S. sanguis*, *S. gordonii*, *S. mitis* biovar I, and *A. naeslundii* strains. See legend to figure 1.

Discussion

In this study we have tested the hypothesis that oral bacteria, physiologically related to *S. mutans*, control the regrowth of the latter organism in dental plaque after intensive chlorhexidine therapy. It is known from clinical studies that long-term suppression of mutans streptococci can be obtained following treatment of the dentition with chlorhexidine gels or varnishes [Emilson et al., 1976; Malz et al., 1981; Zickert et al., 1982; Schaeken and de Haan, 1989; Sandham et al., 1991]. Surprisingly, treatment of a single site of the dentition with chlorhexidine varnish also results in long-term local suppression, despite unaltered high concentrations of mutans streptococci in saliva [Schaeken et al., 1989]. The rapid return of *S. mutans* in the mono-associated rats indicated that the chlorhexidine varnish itself cannot account for long-term suppression. The hypothesis that other microorganisms control the return of *S. mutans* was confirmed by the observations of the prolonged suppression of this species in the rats multi-associated with strains of *S. oralis*, *S. sanguis*, *S. gordonii*, *S. mitis* biovar I and *A. naeslundii*. These strains were isolated earlier from human fissures where they occurred shortly after chlorhexidine varnish treatment. The rapid return of the 'oralis-group' streptococci after chlorhexidine applications is a consistent finding in clinical studies [Schaeken et al., 1989] and is in accordance with the relatively low sensitivity of these organisms to chlorhexidine [Emilson, 1977]. It is noted that recolonization of enamel surfaces after chlorhexidine treatment proceeds according to a pattern similar to that observ-

Fig. 3. *A. naeslundii* strains in two diet groups of multiple-associated rats. See legend to figure 1.

ed for recolonization of cleaned tooth surfaces [Nyvad and Kilian, 1987; Schaeken et al., 1994]. The initial suppression and subsequent regrowth of *A. naeslundii* strains by chlorhexidine treatment were consistent with earlier observations in clinical studies [Schaeken et al., 1989] and in primates [Emilson et al., 1981]. This seems to reflect the high sensitivity of *Actinomyces* to chlorhexidine [Baker et al., 1987]. Competition for growth-limiting substrates is likely to be a major factor in the inhibition of *S. mutans* by other streptococci and actinomyces [van der Hoeven et al., 1985; van der Hoeven and Gottschal, 1989]. Other factors controlling *S. mutans* include its sensitivity to the salivary per-

oxidase system [Carlsson et al., 1983] and its inability to utilize salivary glycoproteins as a source of carbohydrate [van der Hoeven and Camp, 1993].

The more rapid return of *S. mutans* in the group on the sucrose diet than in the basal diet group agrees with the known stimulatory effect of low molecular weight carbohydrates, particularly sucrose, on the number of mutans streptococci in plaque [Krasse et al., 1967; de Stoppelaar et

al., 1970; van Houte et al., 1976; Kilian and Rølla, 1976; Minah et al., 1981; de Jong et al., 1985]. The rats in our experiment were fed ad libitum, which led to a mean number of 15 sucrose-containing meals per 24 h (unpublished observations). It would be of practical importance to know how the frequency of sucrose intake would influence the regrowth of *S. mutans* after chlorhexidine treatment.

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