Colonization of the Human Oral Cavity by a Strain of *Streptococcus mutans*

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Streptococcus mutans *strain JH1001 produces a bacteriocin that can kill virtually all other strains of this micro-organism. The ability of JH1001 to colonize the human oral cavity was tested in a study involving five subjects and three different infection regimens, all of which involved multiple exposures to large numbers of organisms. Two and one-half years after infection, JH1001 was found to have persistently colonized three of the subjects. The indigenous* S. mutans *in one subject were reduced below the level of detection. Levels of (total)* S. mutans *and* S. sanguis *were not affected in persistently colonized subjects. Mutants of indigenous* S. mutans *resistant to the bacteriocin were not observed. The results indicate the importance of host variability and infection regimen for superinfection by this strain of* S. mutans. *The efficient replacement of indigenous* S. mutans *by JH1001 in one subject lends support to the eventual application of replacement therapy to the prevention of dental caries.*

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Introduction.

Prevention of an infectious disease by replacement therapy involves purposefully colonizing a susceptible host tissue with a non-virulent micro-organism. Once established, this so-called "effector strain" must be capable of preventing infection by a pathogen whenever the host comes into contact with it. Several laboratories have studied replacement therapy as an approach for the prevention of dental caries (Hillman, 1978; Tanzer *et al.*, 1982a; Mao and Rosen, 1978; Kishimoto and Morioka, 1981; Fan *et al.*, 1983). A large body of evidence has implicated *Streptococcus mutans* as the principle etiologic agent of this disease (Krasse *et al.*, 1968; deStoppelaar *et al.*, 1969; Littleton *et al.*, 1970). Hence, the majority of effort has gone into isolating non-virulent mutants of this organism to use as effector strains. The results of these studies indicate that there are several ways that the virulence of *S. mutans* can be attenuated without significantly affecting its general nature and, in particular, its ability to colonize the oral cavity.

The major stumbling block to the replacement therapy approach is that the vast majority of humans are persistently infected with *S. mutans* at an early age (Berkowitz *et al.*, 1975; Carlsson *et al.*, 1975). Effective treatment of these individuals requires that the non-virulent effector strain be able to superinfect their teeth and also displace the indigenous strain to the extent that its numbers are insufficient to cause disease. Diverse infection regimens have been used to test a large variety of *S. mutans* strains for their ability to superinfect subjects harboring an indigenous strain of this organism (Krasse *et al.*, 1967; Jordan *et al.*, 1972; Edman *et al.*, 1975; Ruangsri and Ørstavik, 1977; Svanborg and Loesche, 1978; Svanborg and Krasse, 1981; Tanzer *et al.*, 1982b). Far from displacing the indigenous strain, the challenge strain itself was invariably cleared, usually within a span of several weeks.

These results suggest that an *S. mutans* effector strain for use in the replacement therapy of dental caries must have some strong selective advantage to promote its ability to superinfect teeth already colonized by a naturally occurring strain. We have recently reported the isolation of an *S. mutans* strain that produces an unusually potent and broad-spectrum bacteriocin, active against virtually all other strains of this bacterium (Hillman *et al.*, 1984). Mutant analysis strongly suggested that bacteriocin production correlated with colonization potential by this organism in rodents. Also, it was found that a mutant producing two- to three-fold-elevated amounts of bacteriocin could completely displace an indigenous strain of *S. mutans* from the mouths of rats.

The present study was a preliminary attempt to test the ability of this bacteriocinogenic strain of *S. mutans* to colonize the human oral cavity and thereby serve as a starting strain for replacement theory.

Materials and methods.

Micro-organisms and media. — Strain JH1000 has been previously characterized (Hillman *et al.*, 1984). It is a serogroup *c* strain of *S. mutans*. It was isolated from the saliva of a clinically healthy adult, and has all of the metabolic and physiologic properties characteristic of wild-type *S. mutans*. A spontaneous mutant, JH1001, resistant to 1 μg/ml tetracycline (Hillman *et al.*, 1984) was used in the following studies. Broth cultures of strains were grown overnight in Todd-Hewitt medium (Difco) containing 0.5% glucose. *Streptococcus sanguis* and *S. mutans* were recovered from saliva samples on mitis-salivarius agar (MS) and mitis-salivarius agar containing bacitracin (MSB; Gold *et al.*, 1973), respectively. Bacteriocin production was determined on brain-heart infusion agar. Inocula for experiments were taken from starter plates made at weekly intervals from 50% glycerol stabs.

Infection of human subjects. — Five staff volunteers of the Forsyth Dental Center served as subjects. Their indigenous *S. mutans* were isolated from saliva samples by characteristic morphology on MSB medium. The identity of these isolates was tested by their ability to ferment mannitol and sorbitol and to produce adherent plaque on glass and wire surfaces when incubated in the presence of sucrose. Sensitivity of the isolates to the JH1001 bacteriocin was tested as described below. A 100-ml overnight culture of JH1001 (*ca.* 10^9 colony-forming units/ml) was centrifuged and concentrated 20-fold in Todd-Hewitt Broth containing 2% sucrose. Three subjects were treated by brushing and flossing the cell suspension onto their teeth for three minutes. Unattached cells were largely removed by repeated rinsing with water. The procedure was repeated on four consecutive days. The remaining two subjects were treated similarly except for the following changes: Prior to the first exposure to JH1001, their teeth were polished with pumice and a rubber cup. Subject 4 received one infection regimen per day for four consecutive days, and a single re-infection once per week for ten additional weeks. Subject 5 received two exposures to JH1001 per day for four consecutive days.

One to two ml of unstimulated saliva were obtained from subjects at weekly intervals and tested for their content of indigenous *S. mutans* and strain JH1001. The saliva was vig-

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orously vortexed for 30 sec and serially diluted in phosphate-buffered saline (pH 7). Aliquots were spread on MSB and MSB containing 1 μg/ml tetracycline. At intervals, purified isolates of indigenous *S. mutans* and JH1001 were tested by the overlay technique for their sensitivity to and production of bacteriocin, respectively.

Overlay technique for bacteriocin production and sensitivity. — Single colonies of strain JH1001 were stab-inoculated into brain-heart infusion medium (Difco). The plates were incubated for 24 hours in candle jars at 37°C. Three ml of molten agar containing 1 to 1,000 diluted overnight culture of the indicator strain was poured evenly over the surface of the plate. After an additional 24 hours of incubation, the diameters of clear zones surrounding the JH1001 stab were measured.

Results.

All five subjects were found to possess indigenous *S. mutans* that were sensitive to 1 μg/ml of tetracycline and were inhibited in the overlay technique by the bacteriocin of JH1001. Twenty-four-hour stabs of JH1001 caused 1- to 2-cm clear zones in the top agar seeded with the indigenous strains.

Following infection with JH1001, saliva samples were obtained from the subjects at weekly intervals for 18 weeks and analyzed on MSB media with and without tetracycline for their content of indigenous *S. mutans* and JH1001. The efficiency of plating of the indigenous strains and JH1001 on MSB was determined by plating standardized samples on this medium and blood agar medium. No significant differences between the strains could be detected. Of the three subjects who were infected once per day for four consecutive days, two subjects showed a precipitous decrease in the recovery of JH1001 to the extent that the organism could not be recovered 18 weeks post-infection (Fig. 1; < 10 colony-forming units/ml of saliva). The level of JH1001 recovery from the saliva of the third subject decreased to 20% of the total recoverable *S. mutans* within 15 weeks, and remained at that level over the succeeding 2½-year period.

Twenty JH1001 isolates were obtained from subject 3 at weeks 1, 4, 10 and 17 and at 2½ years post-infection. The isolates were purified on MSB medium containing tetracycline, and compared with JH1001 stored in glycerol stabs, by the overlay technique, for their ability to produce bacteriocin. This method revealed no apparent difference between the original starting strain and the recovered isolates. Twenty purified indigenous *S. mutans* isolates recovered 4, 7, and 17 weeks and 2½ years post-infection demonstrated unaltered sensitivity to the JH1001 bacteriocin.

Subject 4 received an infection regimen consisting of a dental prophylaxis followed by exposure to JH1001 once per day for four days, plus single re-infection exposures once per week for an additional 10 weeks. As expected, the proportion of JH1001 remained high throughout the 11-week infection regimen (Fig. 2), and then decreased over the next 10 weeks until it constituted 50% of the total *S. mutans* in saliva. It remained at that level throughout the remaining 2½ years of this experiment.

Subject 5 received a dental prophylaxis followed by two infection exposures per day for four days. There was a rapid decrease in the recovery of JH1001 through the first nine weeks post-infection, followed by a steady rise (Fig. 2). Saliva samples obtained at the conclusion of the experiment revealed that the indigenous *S. mutans* constituted less than 1% of the total *S. mutans* recoverable on MS agar. Plaque samples taken directly from the molar teeth of subject 5 also contained the indigenous organism at levels below this limit of detection.

Mean total *S. mutans* counts (indigenous plus JH1001) in the saliva of subjects 3, 4, and 5 at the conclusion of the experiment did not differ significantly ($p < 0.05$, Student's *t* test) from values for their indigenous strains determined prior to the start of the experiment (Table). *Streptococcus sanguis* isolated from these three subjects had been shown to be sensitive to the JH1001 bacteriocin by the overlay technique. Nonetheless, the levels of *S. sanguis* recovered from the saliva of these subjects also remained unchanged over the course of this experiment.

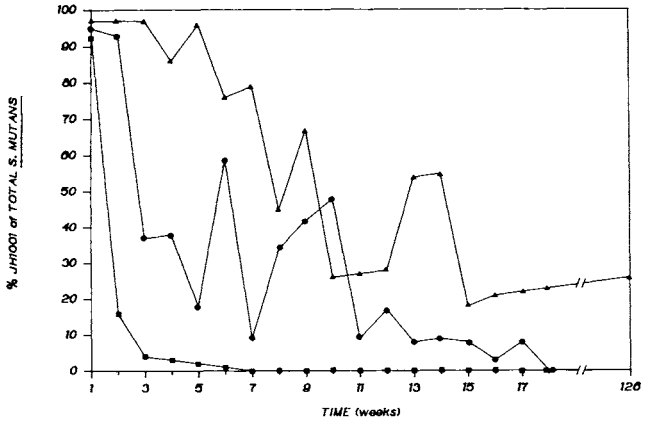

Fig. 1 — The proportion of total *S. mutans* that was JH1001 is plotted against time for subject 1 (squares), subject 2 (circles), and subject 3 (triangles).

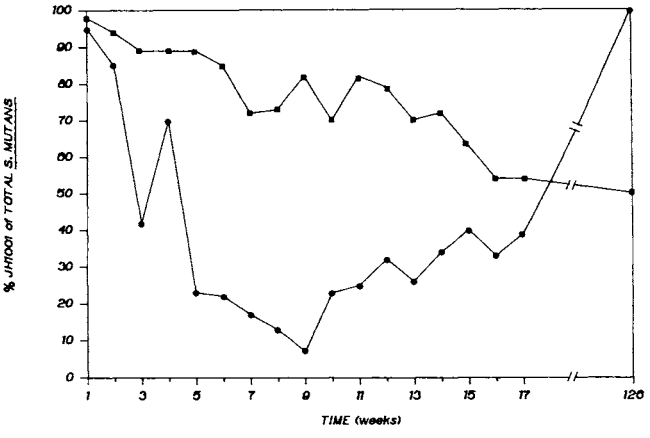

Fig. 2 — The proportion of total *S. mutans* that was JH1001 is plotted against time for subject 4 (squares) and subject 5 (circles).

TABLE
S. mutans AND *S. sanguis* LEVELS BEFORE AND 2.5 YEARS AFTER TREATMENT WITH JH1001

	Mean Concentration in Saliva (CFU × 10^5/ml)*			
	S. mutans (total)		*S. sanguis*	
Subject	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
1	9.4	8.0	—	—
2	1.8	4.2	—	—
3	20.0	17.9	—	—
4	3.0	1.9	11.8	20.4
5	1.2	2.1	34.5	55.4

*Values were determined from a minimum of three independent samples.

Plaque samples taken with a sterile explorer from the mandibular teeth of persistently colonized subjects showed that JH1001 colonized all of the dental surfaces, with a slight predilection for the approximal. Only one of 28 samples taken from subject 4 contained both the indigenous strain and JH1001. The remaining 27 samples contained one or the other, or neither.

Discussion.

Several laboratories have attempted to implant *S. mutans* into the oral cavities of human subjects. In no case has a systematic study been performed to delineate the relative importance of infection regimens and strain and host variability. The results of the present study indicate most clearly the importance of host variability. In the first study, three subjects were infected using an identical regimen. The half-lives of strain JH1001 in two of these subjects were determined, by a nonlinear least-squares method (Marquardt, 1963), to be 4.0 and 1.3 weeks. The third subject remained persistently colonized at the 20% level over the course of 2½ years. There was no apparent correlation between the level of indigenous *S. mutans* infection and susceptibility to superinfection by JH1001.

The importance of the infection regimen is also suggested by the present study. Dental prophylaxis to reduce the numbers of indigenous micro-organisms on the tooth surfaces, and/or increased numbers of exposures to the infecting strain, promoted persistent colonization in the two subjects tested. The relative contributions of these two factors to superinfection would require a systematic study involving a greater number of subjects.

In a previous study (Hillman *et al.*, 1984), JH1001 was found to be greatly superior to *S. mutans* strain Ingbritt in its ability to superinfect and pre-emptively colonize the oral cavities of conventional rats. Although the infection regimens used in this study involved more exposures to higher numbers of cells than did regimens used in other human studies, the ability of JH1001 to colonize and persist in the human oral cavity did not depend entirely on these factors. Similar and even more extensive regimens using *S. mutans* strains BHT-2, Ingbritt, or GS5 failed to produce persistent colonization (data not shown) by these strains.

Indigenous *S. mutans* that had acquired resistance to the JH1001 bacteriocin were not found. This agrees with our inability to isolate resistant mutants of laboratory strains *in vitro* following mutagenesis. It suggests that the target for the JH1001 bacteriocin is essential for viability. This hypothesis is supported by the broad spectrum of activity of the bacteriocin, which indicates that the target is a highly conserved molecule.

Streptococcus sanguis strains isolated from the five subjects were sensitive to the JH1001 bacteriocin. The levels of this organism did not change significantly throughout the course of the experiment. While both *S. sanguis* and *S. mutans* are known to colonize the crowns of teeth preferentially, this finding suggests that they have distinct, non-overlapping niches.

The most significant finding of this study is that extensive displacement of an indigenous strain of *S. mutans* by a superinfecting strain can be accomplished. This finding lends strong support to the eventual application of replacement therapy to the prevention of dental caries. Obviously, a major concern of future studies is to determine the minimal infection regimen necessary to ensure complete displacement of indigenous *S. mutans* by an effector strain. The use of effector strains better able to colonize the human oral cavity (such as the bacteriocin hyper-producing derivative of JH1001) will, it is hoped, result in a minimum infection regimen that is sufficiently practical for use on a routine basis.

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