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Variable Colonization by Oral Streptococci in Molar Fissures of Monoinfected Gnotobiotic Rats

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Germfree Sprague-Dawley rats, fed a high-sucrose diet, were monoinfected with strains of *Streptococcus mitis*, *Streptococcus sanguis*, and *Streptococcus mutans*. Viable cell recoveries from six molar teeth, considered to reflect mainly bacterial colonization of intact fissures, were in the order of 10^6, 10^7, and 10^8 CFU, respectively. Some of the implications of the variation of bacterial plaque-forming ability in the rat model are discussed.

Organisms belonging to the *Streptococcus mutans* group are presently considered as major etiologic agents of human coronal dental caries (9, 14). This view is based on their metabolic traits, their generally consistent ability to induce high caries activity in rodents and other experimental animals, and the positive association of their plaque proportions with human dental caries activity. In contrast, convincing evidence for a major etiologic role of other predominant acidogenic plaque organisms is lacking (9, 14). In particular, representative strains of many of such organisms are often incapable of inducing significant or any caries activity in rodents.

A prerequisite for the expression of bacterial cariogenic potential in rats and hamsters is the formation of dental plaque. This fact has been long recognized with respect to smooth surface caries (14, 16). On the other hand, the inability of test organisms to induce caries in molar fissures of rats has generally not been linked to inadequate plaque formation. The accumulation of bacterial cells in these retentive tooth surface areas has often been assumed to result from their mechanical entrapment. However, the existence of major variability among different bacteria with respect to their plaque-forming ability in rat molar fissures is now suggested by several studies with monoinfected gnotobiotic rats. For example, mean viable cell recoveries, most likely reflecting mainly bacterial colonization in molar fissures, have been found to be a factor of over 30- to over 400-fold lower in the case of two *Bifidobacterium* strains (17) than in the case of *Actinomyces viscosus* T14-Av (3). Very recent studies, involving inspection by scanning electron microscopy of a selected molar fissure, have also indicated major differences among different streptococcal strains with respect to the amount of plaque formed (5, 6). Data in the present report, pertaining to cell recoveries obtained from the occlusal molar surfaces of gnotobiotic rats that were monoinfected with strains of different streptococcal species, further corroborate the findings from these studies.

Germfree Sprague-Dawley rats were infected with different strains of *S. mutans*, *Streptococcus sanguis*, and *Streptococcus mitis*. The *S. mutans* strains were IB-1600 (12), JC-2 (2), and H-2. Strain IB-1600 was a fresh isolate from the teeth of a gnotobiotic rat; strain JC-2 was a laboratory strain, whereas strain H-2 was a fresh isolate from human dental plaque which was lyophilized immediately after isolation and characterized as serotype c by the scheme of Shklair and Keene (13). *S. sanguis* F-43, FC-1, F-41, 34, and NCTC 10904 (4) and *S. mitis* H-1, C-1, S-1, and S-2 were all, with the exception of strains 34 and NCTC 10904, fresh isolates from human dental plaque and were lyophilized immediately after isolation. All these strains were identified by the methods described by Carlsson (4).

Germfree rats were obtained from the Forsyth Dental Center colony. They were provided a low-fluoride diet (L-356; Teklad, Madison, Wis.) until 21 days of age and were then fed diet 2000 (11). After 3 days, they were inoculated with each test strain. Cells for inoculation were obtained from cultures in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) that were incubated anaerobically (80% N_2, 10% H_2, 10% CO_2) in Brewer Anaerobic jars (Becton Dickinson and Co., Paramus, N.J.) for 16 to 18 h at 37°C. The germfree techniques that were employed have been described previously (7).

Rats were sacrificed at the end of each experiment and decapitated. With the aid of a dissecting microscope (×10 magnification), all surfaces of six molar teeth (three molars in the left upper and three molars in the right lower quadrant), except the occlusal surfaces, were cleaned thoroughly by swabbing with cotton-wrapped fine endodontic broaches; a separate broach was used for the three molars in each quadrant of each rat, whereas the use of these broaches also permitted access to the small spaces between the approximating tooth surfaces. Next, the six cleaned teeth from each rat were extracted, pooled, and ground in glass tissue grinders (16). The resulting tooth debris-plaque mixture was then sonicated for 3 min with a microprobe (MSE, Westlake, Ohio) on setting 6 to dislodge cells that remained on the tooth material and to disperse clumps of cells; sonication for 3 min was found previously to yield optimal cell recoveries as determined by microscopic inspection of smears (only single cells with some doublets) and viable counts on blood agar medium. The tooth debris-plaque mixture obtained from each rat was then diluted in sterile saline (serial 10-fold dilution), and samples of 0.1 ml of appropriate dilutions were streaked evenly with a bent glass rod on the surface of blood agar plates with a composition of 4% Trypticase soy agar, 5% sheep blood, and 5% sucrose. The agar plates were incubated anaerobically for 3 days at 37°C, after which the

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TABLE 1. Cell recoveries and cariogenicity of different streptococcal strains in gnotobiotic rats fed a high-sucrose diet

Strain	No. of rats	Test period (mo)	Recovery[a] (CFU/6 molars)	% Fissures at risk with caries category:				
				Total	A	T	B	C
S. mitis H-1	6	1	1.87×10^6 (0.23)	7	7	0	0	0
S. mitis C-1	5	1	2.01×10^6 (0.17)	8	8	0	0	0
S. sanguis F-43	6	1	1.71×10^7 (0.40)	28	28	0	0	0
S. sanguis FC-1	5	1	1.64×10^7 (0.21)	16	16	0	0	0
S. sanguis 34	5	1	1.38×10^7 (0.15)	8	8	0	0	0
S. mutans H-2	5	1	1.06×10^8 (0.14)	45	30	10	5	0
S. mutans IB-1600	5	1	1.70×10^8 (0.51)	32	20	10	2	0
S. mitis H-1	5	3	1.60×10^6 (0.20)	21	19	2	0	0
S. mitis S-1	5	10	7.04×10^6 (0.62)	43	22	11	2	8
S. mitis S-2	6	10	3.63×10^6 (0.66)	38	38	0	0	0
S. sanguis NCTC 10904	6	4	1.79×10^7 (0.55)	73	65	6	2	0
S. sanguis F-41	6	8	6.10×10^7 (1.03)	88	2	8	8	70
S. mutans JC-2	4	2.5	1.44×10^8 (0.18)	100	0	8	18	82
S. mutans IB-1600	6	5	2.01×10^8 (0.44)	98	5	2	5	86

[a] Mean (standard error) of recoveries.

number of colonies on plates with between 50 and 300 colonies was counted; the average counts (five plates per dilution) were used to calculate the total number of CFUs present on the six molar teeth of each rat.

The two jaws with the six molar teeth that were not used for bacterial enumeration were defleshed by dermestid beetles and used for caries evaluation (8). This involved sectioning of the teeth mesiodistally with a diamond disk before staining with Schiff reagent and inspection with a dissecting microscope. The caries categories for the fissures were: A, a lesion where staining is confined to enamel; T, a lesion in which there is staining in dentine but as in the case of the A lesion there is no break in the continuity of the tooth tissue; B, a lesion in which there is staining in the dentine as well as a break in the continuity of the enamel or dentine; C, a lesion which is heavily stained in the dentine with loss of enamel and distinct cavitation. Smooth surface caries was also evaluated at this time on the dry and stained teeth.

According to our past experience and findings of others (1), a test period of 1 month was chosen for a number of experiments. This time period permits the bacterial populations to reach their highest levels in absence of overt cavitation. Findings with seven streptococcal strains are shown in Table 1. The cell recoveries from cleaned molar teeth varied nearly by a factor of 100 among these strains. They were in the order of 10^6, 10^7, and 10^8 CFU for *S. mitis*, *S. sanguis*, and *S. mutans* strains, respectively. One-way analysis of variance of these cell recoveries, after their logarithmic conversion, indicated that they differed significantly ($F = 65.48$, $P < 0.0001$). Differences between strains of different species but not between strains of the same species were also indicated by the Newman-Keuls test for multiple comparisons (5% level). Caries evaluation indicated an absence of cavitation in fissures (only A or T lesions) with the exception of a few fissures in rats infected with the *S. mutans* strains; no smooth surface caries was observed in any of the rats.

Cell recoveries within the same range and of similar variation were observed also among strains tested for longer time periods (Table 1) (one-way analysis of variance, $F = 54.91$, $P < 0.0001$). In addition, the cell recoveries of the same strains of *S. mitis* H-1 and *S. mutans* IB-1600 and of strains of the same species were similar to those obtained at the end of a 1-month period. In spite of the unusually long test period, rats that were infected with the *S. mitis* strains and *S. sanguis* NCTC 10904 developed negligible caries activity. Rats that were infected with *S. sanguis* F-41 and the two *S. mutans* strains developed severe fissure caries (B and C lesions); some smooth surface caries was observed only in the case of *S. mutans* IB-1600.

It seems likely that the cell recoveries from the cleaned rat teeth reflect those from intact fissures, and possibly to some extent from morsal surfaces, in most experiments; furthermore, developing cavitation of teeth may have affected such recoveries in only a few experiments. Collectively, our data and the data from the earlier-cited studies (3, 5, 6, 17) suggest that the extent of cell mass accumulation in fissures varies from negligible to extensive among different bacterial strains under conditions that are commonly used in rat caries studies. Together with electron microscopic observations of the initial phase of bacterial colonization in rat molar fissures (5, 6, 10, 16), these observations first of all appear to minimize the importance of mechanical entrapment of bacterial cells for their accumulation in these tooth surface sites; rather, they support in this respect the significance of cellular adhesion involved in initial cell attachment to the acquired pellicle and subsequent cell accumulation.

Furthermore, as observed by Drucker et al. (5, 6), the plaque-forming ability of all *S. mitis* and most *S. sanguis* strains was greatly inferior to that of the *S. mutans* strains. The reasons for this are not clear. However, several observations are of interest in this regard. First, recent studies by us have shown that extracellular glucan synthesis by *S. mutans* is not a prerequisite for substantial cell accumulation in molar fissures of monoinfected gnotobiotic rats (J. van Houte and J. Russo, International Conference on Cellular, Molecular, and Clinical Aspects of *Streptococcus mutans*, 1985). Thus, the cell recoveries in the case of *S. mutans* IB-1600 and 6715 were found to be in the order of 10^8 CFU/6 molar teeth in the case of sucrose-fed as well as glucose-fed rats. Significant cell accumulation by *S. mutans* C67-1 (serotype c) in molar fissures of sucrose-fed as well as glucose-fed monoinfected gnotobiotic rats was also observed earlier by van der Hoeven et al. (Ph. D. thesis, paper VI, Katholieke Universiteit Nijmegen, Nijmegen, The Netherlands, 1974). This suggests that, although extensive plaque formation by *S. mutans* strains in our studies and the studies by Drucker et al. (5, 6) was most likely mediated by extracellular glucans, *S. mutans* strains also possess mechanisms other than those associated with glucan synthesis for

substantial plaque formation in fissures. Second, our data and the data from Drucker et al. (5, 6) suggest that many *S. sanguis* strains, whether capable of glucan synthesis or not, possess a low plaque-forming ability. In contrast to the *S. mutans* strains, they lack glucan-associated or other mechanisms for substantial plaque formation in fissures. The same appears to be true for the *S. mitis* strains. In view of the evidence supporting a role of salivary components in plaque formation (15), it is possible that *S. sanguis* and *S. mitis* strains lack the ability to adhere to and accumulate on rat molar tooth surfaces by means of adhesive interactions with such components.

Finally, a major issue, not discussed by Drucker et al. (5, 6), concerns the relevance of findings from cariogenicity tests with human plaque organisms in gnotobiotic rats to their cariogenic potential in humans. The cariogenicity of test strains in rats is governed by their plaque-forming ability as well as their capability to acidify the plaque milieu. Consequently, the inferior plaque-forming ability of many potentially important acidogenic human dental plaque organisms in rats prevents adequate assessment of their cariogenic potential in humans. Furthermore, variation of plaque-forming ability among human dental plaque organisms in rats often does not permit a meaningful comparison of their cariogenic potential. Direct extrapolation from findings with rats to the human situation appears unjustified because the colonization of different human acidogenic organisms in rats does not parrallel that of the same types of organisms in human dental plaque (14) where, furthermore, their proportions can vary greatly as a result of varying conditions.

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