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Effect of Microbial Interaction on the Colonization Rate of *Actinomyces viscosus* or *Streptococcus mutans* in the Dental Plaque of Rats

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The resident oral microflora of conventional Osborne-Mendel rats was challenged with *Actinomyces viscosus* or *Streptococcus mutans* strains. The adherence of the inoculated organism to the tooth surface and the subsequent growth were studied by means of viable counts determination. The initial growth rate of *S. mutans* in conventional rats was lower than in mono-associated gnotobiotic rats (doubling time, t_d = 5 h versus t_d = 1.1 h). The delayed start of growth and the low initial growth rate indicated that a competitive interaction between *S. mutans* and the resident microflora occurred. The initial growth rate of *A. viscosus* in conventional rats (t_d = 3.1 h) was approximately the same as that in gnotobiotic rats (t_d = 2.8 h). The start of growth of *A. viscosus* was only slightly delayed compared with the start in gnotobiotic rats. These results suggest a neutralistic relationship between *A. viscosus* and the resident microflora. *A. viscosus* reached a stationary level about 7 days after inoculation, whereas the *S. mutans* strains did not reach stationary levels until 2 weeks after inoculation.

This study deals with the rate at which new bacteria colonize the dental plaque in rats. After inoculation into the mouth, some of the cells attach to the tooth surfaces or to the microbial layer on the teeth. In the next phase the adherent cells start to divide. The rate of increase of the adherent population depends upon the rates of cell division, cell release, cell death, and settling of cells from saliva. During early colonization these latter processes are likely to be of little importance and the population increase will be mainly determined by cell division. At a later stage, during transition to or in steady-state situation, the processes of cell release, cell death, and settling of cells will be of more importance, and calculated doubling times, based on the determination of colony-forming units (CFU) of the population, reflect only the net result of all the involved processes (1).

As the growth rate of bacteria is governed by the concentration of substrates in the environment (12), the rate of increase of the inoculated microorganism in the plaque will provide information on the availability of substrates. Furthermore, the newly entering organism may interact in different ways with the resident microflora, and this interaction may influence its rate of cell division. It is very difficult, however, to obtain experimental evidence for the occurrence of particular microbial interactions in dental plaque, and only in a few cases has such evidence been presented (see reviews by Gibbons and van Houte [9], Bowden et al. [3], and van der Hoeven [16]).

In this study the initial doubling times of strains of *Actinomyces viscosus* or *Streptococcus mutans* are measured in conventional rats. The effect of interaction of the inoculated microorganisms with the resident microflora on the doubling time is evaluated by comparison with the previously estimated doubling time of the same microorganisms in monoinfected gnotobiotic rats.

MATERIALS AND METHODS

Microorganisms. *S. mutans* T2 (13), *S. mutans* C67-1 (5), and *A. viscosus* Ny1 (15) were used in this study. The strains were made resistant to streptomycin (Gist-Brocades N.V.) by sequential inoculation in broth with increasing streptomycin concentrations. No difference was found between the growth rates of the resistant or parent strain in batch culture. The strains were kept lyophilized in stock. Inocula of *A. viscosus* Ny1 were prepared from 16-h cultures in actinomyces broth (BBL Microbiology Systems, Cockeysville, Md.). Inocula of the *S. mutans* strains were prepared from 16-h cultures in brain heart infusion broth (Difco Laboratories, Detroit, Mich.). The bacterial cultures were ultrasonically dispersed with a Branson sonifier, type B-12, twice for 15 s with a 15-s interval and with continuous cooling in ice (Microwave tip, 75-W output

in 10-ml volume). Viable counts of the inocula were estimated on Trypticase soy agar (BBL) plates, supplemented with 1% (wt/vol) yeast extract and containing 100 μg of streptomycin per ml (TS-S agar), and on blood agar plates. The inocula of *S. mutans* T2 and C67-1 and *A. viscosus* Ny1 contained 10^9, 3×10^9, and 2×10^{10} viable cells per ml, respectively.

Animals and treatment. Conventional Osborne-Mendel rats, 35 to 44 days old, were randomly distributed among three groups. The 45 rats of group 1 (five litters of nine rats each) were inoculated with *S. mutans* T2, the 45 rats (15 litters, three rats each) forming group 2 were inoculated with *S. mutans* C67-1, and the 60 rats in group 3 (five litters of 12 rats each) were inoculated with *A. viscosus* Ny1.

Twenty-four hours before the inoculation of the microorganisms, the rats were given a sucrose-containing diet. The diet contained 16% sucrose, 44% wheat flour, 32% skim milk powder, 7% yeast extract, and 1% vegetable oil. The diet was available ad libitum during the experimental period. The rats were individually inoculated with 100 μl of the suitable bacterial suspension applied with a 1-ml hypodermic syringe. A fourth group of 28 rats (from six litters), 32 days old, was not inoculated at all. This group served to study the changes in the indigenous oral microflora after the change from ordinary rat pellets to the sucrose diet for a period of 48 h.

Bacteriological procedures. After inoculation (groups 1, 2, and 3) or after the change of diet (group 4), samples were taken at time intervals as indicated in Fig. 1 and 2. Each sample from groups 1, 2, or 3 consisted of five rats; the samples from group 4 included four rats each. The method used for the determination of the number of bacteria on the rat molar teeth was a modification of the procedure described by van Houte et al. (21). The rats were killed, and the three molars in the left half of the lower jaw were extracted by using a dental probe. The molars were ground in a mortar in 0.5 ml of 0.85% saline. The mortar was rinsed with an additional 0.5 ml of saline, and the suspension was transferred into a sterile tube and dispersed for 30 s with a Kontes E/MC sonifier, provided with a microtip, at maximum output. The undiluted sample and suitable dilutions were plated on TS-S agar plates for the enumeration of the inoculated strains. Total viable counts were estimated on blood agar plates. The blood agar plates were incubated for 48 h, and the TS-S plates were incubated for 72 h, at 37°C in an atmosphere of 91% N_2–5% CO_2–4% H_2.

RESULTS

Growth of the inoculated microorganisms and the doubling times of their populations. The numbers of viable cells of the inoculated strains on molar teeth at increasing time intervals after the inoculation are given in Fig. 1, for all three strains tested. The curves demonstrate several phases in the growth of the inoculated strains. Immediately after inoculation the number of adherent cells decreased. *A. viscosus* Ny1 and *S. mutans* C67-1 were found to decrease for a period of about 6 h, and *S. mutans* T2 decreased for a period of 24 h. Thereafter a rapid increase of the populations of the inoculated strains was observed. This rapid increase lasted up to 48 h for *A. viscosus* and *S. mutans* T2 or C67-1 (Table 1); beyond 48 h the rate of increase declined and the population numbers approached a stationary level. *A. viscosus* Ny1 reached a stationary level at about 7 days after inoculation (Fig. 1C; Table 2). In contrast, *S. mutans* C67-1 and *S. mutans* T2 continued to increase for at least 14 days after inoculation, at which point the latter did not seem to have reached its stationary level yet (Fig. 1A and B; Table 2).

The calculated doubling times of the populations of *S. mutans* T2, *S. mutans* C67-1, and *A. viscosus* Ny1 are shown in Table 1. *A. viscosus* Ny1 attained its maximum rate of increase between 24 and 48 h; the doubling time (t_d) was 3.1 h. *S. mutans* C67-1 reached its maximum rate with a t_d of 4.5 h between 6 to 24 h and sustained rapid growth up to 48 h, with a t_d of 7 h. *S. mutans* T2 showed a maximum growth rate between 24 h and 2 days; its doubling time was 5 h. Beyond 2 days, during the transition to a stationary level *S. mutans* T2, *S. mutans* C67-1, and *A. viscosus* Ny1 showed concomitant increases of t_d.

Changes in the indigenous flora. *Streptococcus bovis* was the dominant organism of the indigenous flora on the tooth surface, ranging between 50 and 95% of the total cultivable flora. A *Veillonella* sp. was highly variable and comprised from 1% to 40% of the total flora. Five additional anaerobic gram-negative rod-shaped species varied between 5 and 20%. In the noninoculated rats (group 4), shifts in the indigenous microflora were recorded after a change from rat pellets to the powdered sucrose diet. During the 48-h period, the population of *S. bovis* tended to increase while the gram-negative species remained fairly constant. The *Veillonella* sp., however, decreased considerably during day 2 (Fig. 2). The effect of the inoculated microorganisms can be summarized as follows. Concomitant with the rise of *S. mutans* C67-1 in the period from 7 to 13 days, there was a highly significant drop in the number of *S. bovis* ($P < 0.001$, analysis of variance). The gram-negative species of the indigenous flora also seemed to be depressed by *S. mutans* C67-1, but the drop was not significant. No effect of *S. mutans* T2 on the *S. bovis* population was observed, which was likely due to the low level of strain T2 during the experimental period (Fig. 1A). A high level of *A. viscosus* did not seem to influence *S. bovis* or the gram-negative species (Fig. 1C). Large fluctuations in the number of *Veillonella* sp. also occurred in the inoculated groups. No correlation between the *Veillonella* sp. population and the inoculated microorganisms was observed.

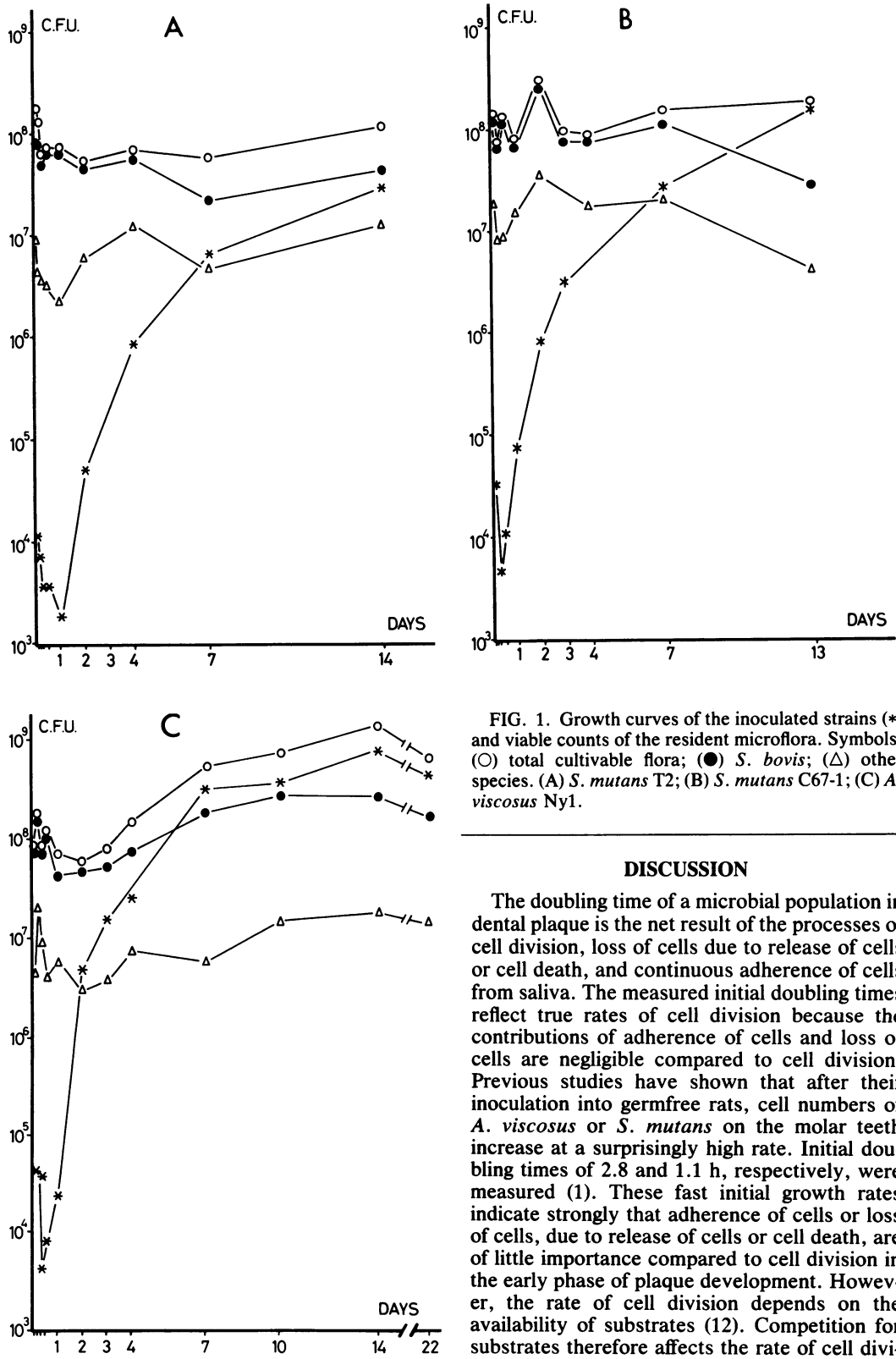

FIG. 1. Growth curves of the inoculated strains (*) and viable counts of the resident microflora. Symbols: (○) total cultivable flora; (●) *S. bovis*; (△) other species. (A) *S. mutans* T2; (B) *S. mutans* C67-1; (C) *A. viscosus* Ny1.

DISCUSSION

The doubling time of a microbial population in dental plaque is the net result of the processes of cell division, loss of cells due to release of cells or cell death, and continuous adherence of cells from saliva. The measured initial doubling times reflect true rates of cell division because the contributions of adherence of cells and loss of cells are negligible compared to cell division. Previous studies have shown that after their inoculation into germfree rats, cell numbers of *A. viscosus* or *S. mutans* on the molar teeth increase at a surprisingly high rate. Initial doubling times of 2.8 and 1.1 h, respectively, were measured (1). These fast initial growth rates indicate strongly that adherence of cells or loss of cells, due to release of cells or cell death, are of little importance compared to cell division in the early phase of plaque development. However, the rate of cell division depends on the availability of substrates (12). Competition for substrates therefore affects the rate of cell divi-

TABLE 1. Doubling times (t_d) of *S. mutans* T2, *S. mutans* C67-1, and *A. viscosus* Ny1 in conventional rats

Strain	Period of increase (t_1–t_2)[a]	t_d[b] (h) ± SE
S. mutans T2	24 h–2 d	5.0 ± 1.1
	2 d–4 d	12 ± 3
	4 d–7 d	27 ± 10
	7 d–14 d	71 ± 12
S. mutans C67-1	6 h–24 h	4.5 ± 1.0
	24 h–2 d	7.0 ± 1.8
	2 d–3 d	13 ± 8
	3 d–7 d	31 ± 12
	7 d–13 d	59 ± 10
A. viscosus Ny1	6 h–24 h	7.3 ± 2.8
	24 h–2 d	3.1 ± 0.2
	2 d–3 d	13 ± 3
	3 d–7 d	25 ± 9
	7 d–14 d	130 ± 50

[a] d, Days.
[b] $t_d = (t_2 - t_1)/3.3 \log (N_1/N_2)$ (N_2 = CFU at time t_2). SE, Standard error.

sion and thus the doubling time of the population.

In conventional rats the slight delay in the start of growth (6 h as compared to 2 h in gnotobiotic rats) and the initial doubling time of *A. viscosus* Ny1, t_d = 3.1 h, suggest the absence of interaction, or neutralism, with the resident microflora. The ultimate level of colonization of *A. viscosus* was higher than that of *S. mutans*. In spite of the predominance of *A. viscosus* the absolute numbers of *S. bovis* or the gram-negative bacteria remained unchanged. These observations support the above suggestion of absence of interaction between *A. viscosus* and the resident microflora.

The initial doubling times of *S. mutans* strains T2 and C67-1 were significantly prolonged compared to the doubling time in gnotobiotic rats (1). The retardation of the initial growth of *S. mutans* can be caused by several mechanisms including decreased attachment, increased release of *S. mutans* cells, antagonistic interactions, or competition for substrates. No evidence is available for decreased attachment or increased release of cells. The initial adherence of *S. mutans* in conventional rats is even higher (70 CFU/10^6 inoculated cells, 2 h after inoculation) than its adherence in gnotobiotic rats fed a sucrose diet (3 CFU/10^6 inoculated cells) or starved (16 CFU/10^6 inoculated cells) (1). Otherwise, there is considerable evidence that sucrose promotes the accumulation of *S. mutans* on the tooth surface (4, 10, 21), so increased release of cells in the presence of sucrose is unlikely. Antagonistic activity of members of the indigenous microflora against *S. mutans* cannot be demonstrated in vitro. Therefore the retardation of the initial growth of *S. mutans* might well indicate a competitive type of interaction between *S. mutans* and members of the resident microflora, in

TABLE 2. Establishment of the inoculated microorganisms

Strain	Days after inoculation	CFU[a] inoculated strain	CFU[a] total cultivable flora	%[b]
S. mutans T2	4	8.5×10^5	6.9×10^7	1.2
	7	6.7×10^6	5.8×10^7	11
	14	2.8×10^7	1.1×10^8	25
S. mutans C67-1	3	3.2×10^6	9.6×10^7	3.3
	7	2.7×10^7	1.6×10^8	17
	14	1.5×10^8	1.8×10^8	83
A. viscosus Ny1	3	1.7×10^7	8.4×10^7	20
	4	2.6×10^7	1.5×10^8	17
	7	2.6×10^8	4.3×10^8	61
	14	6.3×10^8	1.1×10^9	57
	21	3.7×10^8	5.2×10^8	71

[a] CFU per three molars.
[b] (CFU inoculated strain/CFU total cultivable flora) × 100.

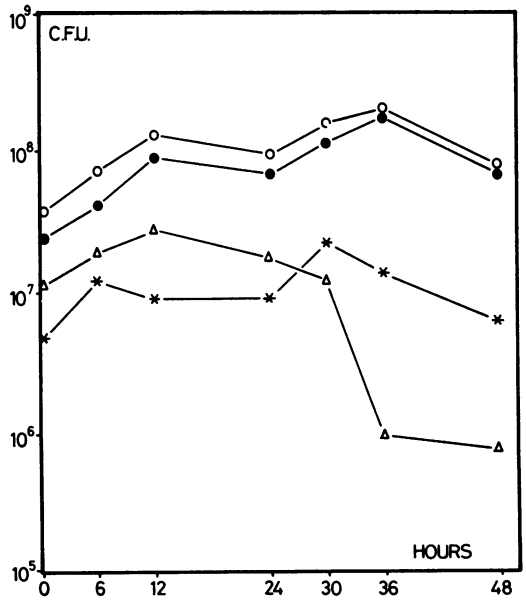

FIG. 2. Viable counts of the resident microflora after change to a powdered sucrose diet. The change of diet was at zero time. Symbols: (○) total cultivable flora; (●) *S. bovis*; (△) *Veillonella* sp.; (*) other species.

TABLE 3. Observed and calculated times of exponential growth and times required to approach the equilibrium state

Strain	Maximum growth rate[a] (h^{-1})	Exponential period (h)		Time required to approach equilibrium (days)	
		$10/D^b$	Observed	$20/D^b - 80/D^b$	Observed
S. mutans T2	0.14	71	48–72	6–24	>14
S. mutans C67-1	0.15	65	48–72	5–20	14
A. viscosus Ny1	0.22	45	48	4–15	7

[a] Maximum growth rate measured ($\mu = \ln 2/t_d$).
[b] Theoretical values from Taylor and LeB. Williams (9).

particular *S. bovis*. Van Houte and Upeslacis (19), studying the sucrose-associated colonization of *S. mutans*, found different growth kinetics for *S. mutans* 6715 in Sprague-Dawley rats, with a fairly long initial doubling time of 8 h as calculated from their data. Moreover, they found that strain 6715 (19, 20) or strain C67-1 (18) attained distinctly lower stationary levels than did strain T2 or C67-1 in our experiments. These differences likely reflect differences between the hosts, Sprague-Dawley or Osborne-Mendel rats, as well as differences in the indigenous microflora of the rats that interact with the inoculated *S. mutans* strain.

From the bacteriological analysis of the plaque, it appeared that *S. bovis* decreased when *S. mutans* became predominant. *S. bovis* and *S. mutans* apparently have overlapping ecological niches, but *S. mutans* is likely to be more competitive. The competition between *S. bovis* and *S. mutans* further seems to be reflected by the delayed start of the growth of *S. mutans* after its inoculation. The delay was significant in particular for strain T2, which was also distinctly affected in its doubling time. Little information is available on the type of interaction between *S. mutans* and *S. bovis*. In vitro, *S. bovis* is inhibited by bacteriocins produced by the strains C67-1 and T2. It is likely that these bacteriocins are also effective in dental plaque (17), which could explain the suppression of *S. bovis*. Bacteriocin action, however, could not account for the increased doubling times of *S. mutans*. Other interactions such as competition for substrates might be involved here. A similar competitive interaction might occur between *S. mutans* and *S. sanguis*, as indicated by the inverse relationship that has been observed between these species in human or rat dental plaque (6, 16a). In other natural ecosystems, the establishment of an invading microorganism depends on the composition of the resident microflora, as was shown by Freter (8) in the gut. Meynell (11) or Ducluzeau et al. (7) demonstrated in the mouse gut the antagonistic effect of the indigenous microflora or specific members of it. Retardation or inhibition of initial bacterial growth of two of the three inoculated bacterial strains was observed by Bennet and Lynch (2) in the rhizosphere of gnotobiotic plants. The suppression of the gram-negative species of the resident microflora of rats might be due to the production of bacteriocin by *S. mutans* (17), but this was not further investigated.

Pronounced differences were observed in the time needed by *A. viscosus* or *S. mutans* to attain a stationary level. *A. viscosus* reached a stationary level at about 7 days after inoculation, and *S. mutans* C67-1 took 14 days; *S. mutans* T2 likely did not reach a stationary level during the experimental period of 2 weeks. These data are in accordance with earlier data obtained in our laboratory on the establishment of *S. mutans* in the plaque of conventional rats (17). However, in those experiments no information was obtained on initial doubling times of *S. mutans*.

A theoretical treatment of the growth of mixed cultures in a chemostat was given by Taylor and LeB. Williams (14). They argued that rapid exponential growth with little interaction between the species occurs initially. After exponential growth is halted, at a time of about $t = 10/D$ (D = dilution rate), because substrate concentrations limit the growth, there is a relatively slow adjustment of the species concentrations towards equilibrium values. The time required to approach the equilibrium state varies between about $20/D$ and $80/D$, depending on the ratios in the inoculum.

Substitution for D of the maximum growth rates determined in this study for the different populations confirmed that the kinetics of the growth of *A. viscosus* or *S. mutans* in dental plaque corroborate those of the theoretical model. The observed and calculated periods of exponential growth and the times required to approach the equilibrium state for *A. viscosus* or *S. mutans* are shown in Table 3.

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