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Role of Interbacterial Adherence in Colonization of the Oral Cavities of Gnotobiotic Rats Infected with *Streptococcus mutans* and *Veillonella alcalescens*

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The role of interbacterial adherence in the colonization of the rat oral cavity was investigated with aggregating and nonaggregating strains of *Veillonella alcalescens* and *Streptococcus mutans*. *V. alcalescens* V-1 and *S. mutans* M-7 rapidly formed large stable aggregates when mixed in vitro. Aggregates could be reduced in size by sonication, but they could not be completely dispersed, indicating that bonding between the organisms was strong. *V. alcalescens* V-1 did not coaggregate with *S. mutans* C67-1, and *V. alcalescens* OMZ193 did not coaggregate with either *S. mutans* strain C67-1 or M-7. Osborne-Mendel rats monoassociated with either *S. mutans* C67-1 or M-7 were inoculated with veillonellae, molar teeth were removed at 2 h and at 14 days, and the number of veillonellae was determined. At 2 h post-inoculation there were 600 times as many colony-forming units of *V. alcalescens* V-1 adherent to the teeth of animals monoassociated with *S. mutans* M-7 when compared with animals monoassociated with the nonaggregating *S. mutans* C67-1. The number of colony-forming units of *V. alcalescens* V-1 was 1,000 times greater than the number of nonaggregating *V. alcalescens* OMZ193 in *S. mutans* M-7-infected animals. Similar results were obtained when teeth were sampled 14 days after inoculation. Veillonellae inoculated into the mouths of germfree animals rapidly disappeared from tooth surfaces.

Once a layer of bacteria has attached to the pellicle on a tooth surface the subsequent accumulation of microorganisms is dependent on bacterium-bacterium binding reactions. In some instances, this will be a result of intrabacterial association in which attachment of the same species may be mediated by glucan (7) or constituents of saliva (9). Interbacterial binding also occurs and is exemplified by the "corn cob" (11) and "test tube brush" (13) structures which can be observed microscopically in human plaque. Presumably, other less obvious interactions occur, and indeed Gibbons and Nygaard (8) demonstrated that many pairs of oral bacteria will aggregate when mixed in vitro. Recently McCabe (14) reported that *Streptococcus mitis* aggregated with *Veillonella* sp. via dextran produced from cell-associated glucosyltransferase. *Veillonella alcalescens* can bind to *Streptococcus salivarius* by a mechanism not related to dextran (24, 25). Other interbacterial interactions have been reported for *Actinomyces viscosus-Streptococcus sanguis* (2, 6, 15) and *Fusobacterium-S. sanguis* (12; B. C. McBride, T. King, T. Edwards, and M. Gisslow, J. Dent. Res. [special issue] **56**:A156, 1977.

Veillonellae are considered to have a moderating effect on caries (16, 17) by raising the pH when lactate is metabolized to weaker organic acids. Mikx et al. (17) found that germfree rats infected with *Streptococcus mutans* and *V. alcalescens* developed fewer caries than did animals monoinfected with *S. mutans*.

In this paper we report on the role of interbacterial binding in the colonization of plaque by *V. alcalescens* in rats monoassociated with *S. mutans*.

MATERIALS AND METHODS

Bacteria. *S. mutans* M-7 was isolated from the human oral cavity on MS-bacitracin medium (19). *V. alcalescens* V-1 was isolated from the human oral cavity on veillonella agar (18). *S. mutans* C67-1 and *V. alcalescens* OMZ193 were obtained from the Nijmegen culture collection. The streptococci were maintained on blood agar, and the veillonellae were maintained on veillonella agar. Streptococci required for adherence studies were harvested from an overnight culture grown in brain heart infusion broth supplemented with glucose (2%). Veillonellae were harvested from an overnight culture grown in veillonella broth and incubated anaerobically in an N_2-H_2-CO_2 (85:10:5) atmosphere. Radiolabeled veillonellae were grown in

veillonella medium supplemented with [U-^{14}C]lactic acid (Amersham Corp; 5 μCi/50 ml).

Aggregation assays. Cells harvested from an overnight culture were washed two times with phosphate-buffered saline and a third time with N-2-hydroxyethyl-1-piperazine-N'-2-ethanesulfonic acid (HEPES) buffer (0.05 M, pH 7.0) and resuspended in HEPES to give a final absorbance at 660 nm of 2.0. Aggregation assay mixtures containing 0.1 ml of each organism were shaken at room temperature; results were scored visually on a 0 to 4 basis. Controls in which one of the organisms was replaced with HEPES buffer were run concurrently. Aggregation was also determined spectrophotometrically by measuring the decrease in absorbance at 600 nm resulting from the settling of microbial aggregates. In the second assay 0.5 ml of each cell suspension was mixed in a 1.0-ml disposable cuvette, removed from the shaker, at designated intervals, and allowed to stand for 5 min, and the absorbance was recorded. Salivary aggregation was measured as described previously (25).

HA binding. Binding to hydroxyapatite (HA) and saliva-coated HA was measured as described previously (24).

Saliva. Saliva was collected from Osborne-Mendel rats which had been anaesthetized with an intramuscular injection of Hypnorm (Philips-Duphar B. V., Amsterdam, Holland; 0.1 to 0.2 ml/100 g of body weight) and then injected subcutaneously with Carbacholum (0.01 mg/100 g of body weight). Saliva was collected on ice and centrifuged at 15,000 $\times$ g for 15 min, and the supernatant was stored at -70°C. Heated saliva was incubated at 60°C for 30 min before storing at -70°C.

Rat studies. In vivo experiments were performed with 7-week-old germfree Osborne-Mendel rats maintained in Macrolon cages (without bedding). One week before inoculation the diet was changed from pellets to a powdered diet containing glucose (516G) (17). The animals were divided into six separate experimental groups. Groups I and II received 0.1 ml of an overnight culture of *S. mutans* M-7, and groups III and IV received 0.1 ml of an overnight culture of *S. mutans* C67-1. Groups V and VI were maintained in a germfree state. One week after inoculation with the streptococci, all groups were inoculated with 0.1 ml of the appropriate strain of *V. alcalescens*. The inoculum was obtained from a culture which had been grown overnight anaerobically in veillonella broth. The culture was centrifuged in the growth tube; 80% of the supernatant was removed aseptically, the pellet was resuspended in the remaining medium, and air was replaced aseptically with nitrogen. *V. alcalescens* V-1 was inoculated in groups I, III, and V, and *V. alcalescens* OMZ193 was inoculated in groups II, IV, and VI. All inocula were delivered into the oral cavity with a sterile syringe.

At the appropriate times, animals were sacrificed by decapitation, and the lower jaw was removed. The three lower left molars were extracted and ground in a mortar and pestle in 1 ml of reduced transport fluid (21). The mixture was placed in a 1-ml Eppendorf centrifuge tube, sonicated for 30 s (Kontes K881440 cell-disruptor), diluted in reduced transport fluid, and immediately plated on veillonella agar which had been incubated in an anaerobic environment until just before use. Immediately after plating the plates were placed in an anaerobic chamber (Coy Manufacturing Co., Ann Arbor, Mich.). Samples were also plated on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) and incubated in the chamber. In a preliminary study, grinding, sonication, diluting, and plating were carried out in the anaerobic chamber, and the number of colony-forming units (CFU) was compared with that of samples treated as described above. There was not a significant difference in the number of CFU of veillonellae; therefore, the technically more simple aerobic procedure was adopted.

Effect of saliva. An overnight culture of veillonellae was centrifuged, and the pellets were suspended in rat saliva which had been shaken in an O_2-free environment for 1 h. The cell suspension was incubated in an N_2-H_2-CO_2 (85:10:5) environment for 2 h at 37°C. The number of CFU was determined at zero time and at 2 h. Controls were run with cells incubated in buffer.

RESULTS

Interbacterial aggregation. Mixtures of *S. mutans* M-7 and *V. alcalescens* V-1 formed aggregates which were evident macroscopically 30 s after mixing (Table 1). Within 5 min 98% of the cells were present in large aggregates which sedimented rapidly; Gram stains of the reaction mixtures revealed that virtually all of the veillonellae were bound to streptococci. The controls incubated with buffer remained as homogenous single cell suspensions. The aggregates were held together tightly and could not be separated by vigorous shaking. Sonication for 45 s reduced the size of the aggregates to the point where they were not visible macroscopically, but microscopic examination revealed that most of the veillonellae remained associated with streptococci either as small clumps or pairs of cells. The sonicated suspensions rapidly reformed large aggregates. *S. mutans* M-7 did not aggregate with *V. alcalescens* OMZ193, and *S. mutans* C67-1 did not aggregate microscopically or macroscopically with either strain of *V. alcalescens*.

Saliva obtained from Osborne-Mendel rats did not induce aggregation of either strain of *V. alcalescens*. Saliva did not inhibit coaggregation of *V. alcalescens* V-1 with *S. mutans* M-7 and actually appeared to stimulate the reaction slightly when both organisms were suspended in saliva before mixing. Coaggregation of the other *Streptococcus-Veillonella* combinations was not induced by saliva.

HA binding. Approximately equal numbers of both strains of *V. alcalescens* bound to uncoated hydroxyyapatite (Table 1). Treatment of HA with saliva reduced the veillonella binding capacity by 85%, probably because constituents in saliva block the HA-veillonella binding sites.

TABLE 1. *In vitro aggregation and adherence properties of V. alcalescens V-1 and OMZ193*

V. alcalescens strain	Interbacterial aggregation[a]						% of cells bound to HA[b]	
	S. mutans M-7		*S. mutans* C67-1		Rat saliva			
	Score	% Decrease in A_{660}	Score	% Decrease in A_{660}	Score	% Decrease in A_{660}	Uncoated	Saliva coated
V-1	4[c]	98	0	2	0	3	72	14
OMZ193	0	0	0	4	0	6	65	10

[a] Interbacterial aggregation assay mixtures contained 0.1 ml of an *S. mutans* cell suspension (5×10^8 cells per ml) and 0.1 ml of a *V. alcalescens* cell suspension (5×10^8 cells per ml). Salivary aggregation mixtures contained 0.1 ml of rat saliva and 0.1 ml of a *V. alcalescens* cell suspension (5×10^8 cells per ml). A_{660}, Absorbancy at 660 nm.

[b] The *V. alcalescens* V-1 and OMZ193 cell suspensions were adjusted to the same cell density as determined by microscopic count in a Petroff-Hauser counting chamber. Final concentration of cells was 5.0×10^8/ml. The percentage reflects the number of cells present on the beads after three washings.

[c] Aggregation measured 5 min after mixing. All other samples were assayed 30 min after mixing the two organisms.

Adherence in vivo. To determine whether the aggregating properties of the M-7–V1 system observed in vitro influenced colonization of teeth, germfree and monoassociated Osborne-Mendel rats were inoculated with aggregating and nonaggregating strains of *V. alcalescens*. One week before introduction of veillonellae, one group of rats was infected with *S. mutans* M-7, another was infected with *S. mutans* C67-1, and a third group was left in the germfree state. Colonization is a complex process involving the ability of bacteria to adhere and grow; these two events were differentiated by measuring the number of bacteria on the tooth 2 h and 14 days after inoculation. Two hours was chosen as an appropriate time to analyze for adherence because this was a period of time sufficiently long to allow the cleansing mechanisms of the mouth to remove unattached microorganisms and yet short enough so that cell division would not influence the results (Bechers and van der Hoeven, unpublished data).

Approximately 300 times more *V. alcalescens* V-1 cells bound to teeth populated with *S. mutans* M-7 than bound to teeth populated with *S. mutans* C67-1. Even fewer *V. alcalescens* V-1 could be recovered from teeth which had no microbial plaque (Table 2). The greater number of V-1 cells was not likely due to increased surface area associated with plaque formation because there were approximately three times more C67-1 than M-7 CFU and yet there were fewer V-1 cells associated with C67-1 plaque. The adherence of *V. alcalescens* OMZ193 was not influenced by the presence of either of the streptococci; approximately equal numbers were found on bacteria-free and bacteria-coated teeth. The specificity of microbial binding was indicated by the fact that the number of *V. alcalescens* V-1 CFU associated with *S. mutans* M-7 plaque was more than 1,000 times greater than the number of *V. alcalescens* OMZ193 bound to the streptococci; if nonspecific trapping were responsible the numbers should have been equivalent. Differences were not due to sensitivity to rat saliva as the viability of either *V. alcalescens* strain was not affected by anaerobic incubation in rat saliva for 2 h at 37°C.

The TS and veillonella agar plates from V-1–M-7-infected animals were populated by a number of colony types which proved on microscopic examination to be pure cultures of the organisms and mixed cultures containing various numbers of the two species. This observation correlates with in vitro studies which showed that sonication did not dissociate aggregates into single cells. Mixed colonies were not observed in samples from the other experimental groups.

Another way of looking at the adherence data is to determine the percentage of organisms in the inoculum which were able to bind to the teeth. When corrected for the inoculum size, it was found that 0.1% of *V. alcalescens* V-1 cells were bound to teeth populated with *S. mutans* M-7, whereas only 0.0003% were bound to *S. mutans* C67-1-coated teeth. The number of CFU of *V. alcalescens* OMZ193 was 1 order of magnitude less than the V-1–C67-1 combination.

Veillonellae are anaerobic organisms and are subject to both the cleansing activity of the oral cavity and to O_2. The fate of the two veillonella strains inoculated into the mouths of germfree animals was followed over a period of 6 h (Fig. 1). The two organisms were found to behave in a similar way; only a small percentage of the inoculum was recovered in the 30-min sample, and this decreased steadily during the 6-h experimental period. The small number of cells

TABLE 2. *Streptococci and veillonellae adherent to the molar teeth of Osborne-Mendel rats 2 h after inoculation with veillonella*

Organism on tooth at time of inoculation	No. of CFU[a]			
	S. mutans	*V. alcalescens* V-1	*S. mutans*	*V. alcalescens* OMZ193
None		6.0×10^3		2.0×10^3 (1.5–2.6)
S. mutans M-7	3.3×10^8 (2.1–4.7)	8.9×10^6 (7.2–10.1)	2.9×10^8 (2.6–3.3)	2.8×10^3 (1.1–3.8)
S. mutans C67-1	2.6×10^9 (2.3–2.8)	2.4×10^4 (1.8–2.8)	1.5×10^9 (1.2–1.9)	6×10^2 (2–9)

[a] Average of three animals; the figures in parentheses are the range of CFU within each group; 1.03×10^8 CFU of *V. alcalescens* V-1 and 6.7×10^8 CFU of *V. alcalescens* OMZ193 were inoculated per ml.

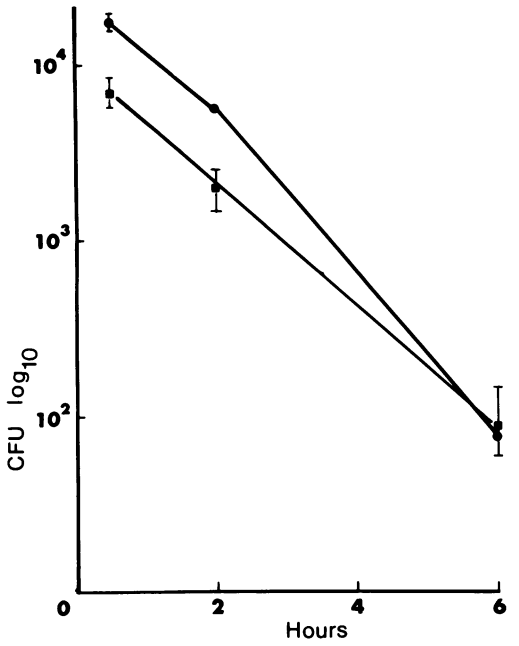

FIG. 1. *CFU of V. alcalescens OMZ193 (■) and V-1 (●) of monoassociated rats during the first 6 h postinfection.*

found on the teeth was probably due to the inability of the cells to adhere well to pellicle-coated teeth and to killing by exposure to O_2. There does not appear to be any difference in the sensitivity of the two strains to O_2, thus eliminating this as a possible explanation for finding fewer *V. alcalescens* OMZ193 cells bound to the *S. mutans* plaque.

Colonization. At 14 days after inoculation of *V. alcalescens* V-1 and OMZ193 into rats monoinfected with either *S. mutans* M-7 or C67-1, the animals were sacrificed, and the numbers of bacteria adherent to the teeth were determined (Table 3). The same trend was observed as was noted for adherence. Animals associated with M-7–V-1 had a *V. alcalescens* V-1 population 3 orders of magnitude greater than that in animals infected with the C67-1–V-1 combination. Animals colonized with *S. mutans* M-7 had an average of 1.0×10^8 *V. alcalescens* V-1 CFU on the three molars, whereas animals colonized with *S. mutans* C67-1 had 3×10^4 *V. alcalescens* V-1 CFU. The *V. alcalescens* OMZ193 population was 1×10^4 and 5×10^4 CFU in *S. mutans* M-7- and C67-1-infected animals, respectively. Comparison of the numbers of *V. alcalescens* V-1 with OMZ193 in the *S. mutans* M-7-colonized rats shows that the V-1 strain comprised 24% of the total plaque microflora, whereas the OMZ193 strain accounted for only 0.035% of the bacterial population. As noted previously, isolated colonies from V-1–M-7-infected animals were frequently mixed cultures of the two organisms.

TABLE 3. *Streptococci and veillonellae adherent to the molar teeth of Osborne-Mendel rats 14 days after inoculation with veillonellae*

Group	No. of CFU[a]	
	S. mutans	*V. alcalescens*
M-7–V-1	3.1×10^8 (1.1–4.4)	1.0×10^8 (0.7–2.0)
M-7–OMZ193	2.8×10^8 (0.9–5.0)	1×10^4 (0.8–2)
C67-1–V-1	1.6×10^9 (0.8–2.1)	3×10^4 (1–5)
C67-1–OMZ193	1.4×10^9 (1.1–3.1)	5×10^4 (4–6)

[a] Average of two animals; the figures in parentheses are the range of CFU.

DISCUSSION

The presence of an array of immobilized receptors on a tooth surface is an important ecological characteristic which will determine whether an incoming organism can locate on the surface before it is washed away. The colonization of rat molars by *V. alcalescens* V-1 can be attributed directly to components on the surface of the microorganism which recognize and bind to receptors in the *S. mutans* cell wall. The large differences observed in the number of adherent organisms within the different experimental groups is a reflection of both the strength of bonds formed between the *S. mutans* M-7 and *V. alcalescens* V-1 and the speed with which the interaction occurs. In vitro analysis indicated that M-7 and V-1 cells formed large aggregates

seconds after being mixed. The ramification of this property in vivo would be to increase the likelihood that random collisions of the incoming organism with the receptors on the resident plaque flora would lead to interbacterial bond formation. Microscopic analysis of sonicated aggregates formed in vitro and the high incidence of mixed colonies in cultures of plaque suggest that the bonds formed between the organisms are strong and would be resistant to the hydrodynamic and mechanical shearing forces operating in the mouth. The strength of the bond implies a low dissociation constant; consequently, the reversibility (10) of the reaction would be minimized. This would be particularly important during the early stages of infection when the potential inoculum of nonassociated organisms is rapidly diminished by salivary dilution and clearing. Thus, the ability to form strong bonds rapidly would favor the accumulation of *V. alcalescens* V-1 on a tooth populated with *S. mutans* M-7.

Saliva bathes all surfaces in the oral cavity and has been postulated to be an important factor in promoting and inhibiting adherence (4, 5, 9, 20). Clark and Gibbons (5) have reported that saliva inhibits binding of *S. mutans* to saliva-coated HA, presumably by occupying receptor sites on the bacterial cell surface. In vitro analysis suggests that the V-1–M-7 system is not inhibited by rat saliva and in fact may be stimulated. Although it is not known how the saliva functions in vivo, the results indicate that there is not a pronounced inhibitory effect in this system.

Colonization of a particular ecosystem is a complex process involving consideration of the growth rate of the organisms, the response of the organisms to body defenses, and the interaction with the resident microflora (1, 3). The multiplicity of factors makes it difficult to measure the influence of a specific property in the colonization process. It is likely that the various factors can be arranged in a continuum of importance. The relative importance of a particular property, i.e., a specific adherence mechanism, will vary from organism to organism and will be influenced by the environment. Notwithstanding these problems, it seems likely that the greater number of *V. alcalescens* V-1 CFU present 14 days after inoculation into *S. mutans* M-7-colonized rats can be attributed to the specific aggregation characteristics of these two organisms. Growth rate could also be a factor, but this seems unlikely because the *V. alcalescens* V-1 population in the nonaggregating *S. mutans* C67-1-infected animals was 3 orders of magnitude less than that in the *S. mutans* M-7-infected animals. Even if the growth rate of veillonellae in the other groups was one-half that of the V-1–M-7-infected animals, 14 days should have allowed sufficient time for the organisms to increase their numbers over that observed 2 h after inoculation.

The concept of a critical inoculum size was illustrated by the observations of van Houte and Green (23) that a minimum number of cells was required to successfully establish an organism in plaque. The ability to rapidly form strong bonds with an immobilized surface would have the effect of reducing the number of cells required to establish a continuing infection. This implies that the minimum infective dose of *V. alcalescens* V-1 in animals colonized by *S. mutans* M-7 would be low.

The rapid disappearance of veillonellae from the tooth surface 6 h after inoculation of monoinfected animals can be related to the inability of the organism to bind to saliva-coated HA and to its sensitivity to O_2. These results are consistent with the in vitro studies of Slots and Gibbons (20), who found that *Bacteroides assacharolyticus* was able to coaggregate with a number of gram-positive organisms, but had a limited capacity to bind to pellicle- or saliva-coated epithelium. They suggested that in the formation of plaque, gram-negative organisms colonize after gram-positive organisms because the latter possess the necessary receptor sites which are either not present or not available on human oral tissue. In addition, single cells attached to a plaque-free tooth surface would be particularly vulnerable to O_2, whereas cells binding to plaque would be protected by the low redox environment created by the streptococci.

Veillonellae have been shown to reduce the caries activity in animals diassociated with *S. mutans* and *V. alcalescens* (17, 22) by metabolizing lactic acid to weaker acids. It would be interesting to determine if diassociation with tightly adhering veillonellae would result in greater consumption of lactic acid due to increased numbers of the organism and to the close association with the lactate-producing cells.

An important aspect of this study was the observation that adherence characteristics defined by in vitro analysis were substantiated by in vivo analysis in the more complex oral environment. For example, the small number of veillonellae found on the pellicle-coated tooth surface of germfree rats correlated with the inability of the organism to attach to saliva-coated HA. Similarly, the interbacterial aggregating properties of the organisms were reflected in the numbers of veillonellae found adherent to *S. mutans* plaque.

The animal model system described in this

report accentuates the differences between aggregating and nonaggregating strains because it creates a plaque consisting of a single species which will create a high concentration of a specific receptor. Organisms unable to use this or other receptors on the bacterial surface will be discriminated against. In a natural plaque containing many different species, there will be a greater variety of receptors and a dilution of any one specific receptor. This means that differences in adherence due to a specific receptor might not be as obvious and, conversely, that other receptor-binding reactions may become important and thus tend to reduce differences in numbers. The advantage and justification of the model is that it can be used to unequivocally define how interbacterial aggregation influences colonization by magnifying differences between microorganisms. The same events occur in conventional animals, but other binding systems will be operating and will make it difficult to separate out and measure the reaction of interest.

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