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Selective Bacterial Adherence to Oral Epithelial Surfaces and Its Role as an Ecological Determinant

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The possible relationship between the ability of *Streptococcus* species to adhere to oral epithelial cells and their natural distribution on epithelial surfaces within the mouth was studied. Strains of *S. salivarius* and *S. sanguis*, which are present in significant proportions on oral epithelial surfaces, were found to possess a definite capacity to adhere to epithelial cells obtained from cheek scrapings of humans, hamsters, and germ-free rats. In contrast, strains of *S. mutans*, which are found in only minor proportions, if at all, on oral epithelial surfaces exhibited feeble or no adherence to oral epithelial cells. *S. salivarius* cells attached well to human cheek cells over the range of *p*H 5 to 8. Its adherence was not found to be markedly influenced by saliva or by growth in sucrose broth. Several other types of bacteria were examined which also exhibited widely different abilities to attach to human cheek cells. Mixtures of streptomycin-labeled strains were introduced into the mouths of volunteers for study of the adherence of *Streptococcus* species to oral epithelial surfaces in vivo. Labeled *S. salivarius* and *S. sanguis* were recovered in high proportions from cheek and tongue surfaces, whereas the proportions of labeled *S. mutans* recovered from these surfaces were low in comparison to the original mixture. These data indicate that a correlation exists between the relative adherence of various *Streptococcus* species and their proportional distribution found naturally on oral epithelial surfaces. The ability of bacteria to adhere to surfaces is proposed as a critical ecological determinant affecting their colonization in environments with open surfaces exposed to bathing fluids.

It is widely recognized that qualitative and quantitative differences exist in the composition of the microbial flora colonizing various sites within the human oral cavity. This selective localization of bacteria is especially evident among those species of oral streptococci which are readily identifiable. For example, the streptococcal populations of microbial plaques developing on the surfaces of teeth characteristically exhibit high percentages of *Streptococcus sanguis* but low proportions of *S. salivarius* (2, 3, 10, 14). Under the influence of dietary sucrose, high proportions of *S. mutans* may also be found (16). In contrast, the oral epithelial surfaces harbor significant proportions of *S. salivarius* and *S. sanguis*, and, in fact, *S. salivarius* averages over 50% of the streptococci present on the tongue (3, 10, 14). However, *S. mutans* is infrequently recovered in large proportions from the tongue and cheek. The reasons for this striking bacterial localization in various sites in the mouth have never been clear. Frequently, this has been vaguely attributed to possible differences in nutrient availability in the respective microcosms. Recently, *S. salivarius* and *S. sanguis* have been found to possess markedly different abilities to adhere to the surfaces of teeth, and it has been suggested that the reason *S. salivarius* is not abundant on teeth is due to its relatively poor adherence in this site (22). This selective ability of bacteria to attach to surfaces could therefore be a major ecological determinant. If this hypothesis is correct, then *S. salivarius*, which is abundant on the oral epithelial surfaces, should be able to adhere well to such surfaces, whereas *S. mutans* should not. The present investigation describes the selective abilities of bacteria to attach to epithelial cells and indicates that the relative adherence of oral streptococci correlates with their natural occurrence on oral epithelial surfaces.

MATERIALS AND METHODS

Cultures and cultural conditions. Laboratory strains of *S. mutans*, *S. salivarius*, *Actinomyces viscosus*, *A.*

naeslundii, *Streptococcus* strain 26, *S. faecalis*, and *Escherichia coli* have been previously characterized and were obtained from the culture collection of the Forsyth Dental Center. New strains of *S. sanguis* and *S. salivarius* were isolated from human saliva, and strains of fusobacteria, *Neisseria*, *Nocardia*, and *Rothia* were isolated from human tooth surfaces. *Streptococcus* strain B1 was isolated from oral epithelium. All organisms were maintained by weekly transfer in Brewer thioglycollate broth (Difco). Cell suspensions of each organism were prepared from 24- to 48-hr Trypticase soy broth (BBL) cultures which were incubated anaerobically at 35 C in Brewer Jars filled with 80% N_2, 10% H_2, and 10% CO_2. The organisms were harvested by centrifugation, washed once with 0.067 M phosphate buffer at *p*H 6.0, and finally suspended in buffer to yield a cell suspension having an optical density at 550 nm of 0.6. In the case of the streptococci studied, these suspensions contained from 10^8 to 3×10^8 organisms per ml as determined by direct microscopic count. The possible influence of saliva on the ability of *S. salivarius* and *S. mutans* to adhere to epithelial cells was studied by suspending the organisms in freshly collected human saliva which had been clarified by centrifugation at $12,000 \times g$ for 10 min.

Preparation of epithelial cell suspensions. Human epithelial cells were collected by scraping oral mucosal surfaces with a wooden applicator stick. The applicators were twirled in saline to dislodge the cells. Suspensions obtained from three to six subjects were pooled together. The cells were washed free of unattached bacteria by using membrane filters having a pore size of 14 μm (Millipore Corp., Bedford, Mass.). A weak vacuum was applied to the filter, and the cell suspension was constantly agitated with a rubber-tipped glass rod. When the volume of the suspension decreased to 1 or 2 ml, fresh saline (10 ml) was added. The entire washing procedure was repeated four to five times. The washed epithelial cell suspension was adjusted with saline to contain 10^5 cells per ml as determined by microscopic count. Some experiments employed cheek epithelial cells obtained in a similar manner from conventional hamsters or from germ-free Sprague-Dawley rats.

Epithelial cell-bacteria reaction mixtures. The ability of various bacteria to adhere to epithelial cells was studied by mixing 1-ml samples of standardized suspensions of bacteria and epithelial cells together. The reaction mixtures were incubated in a shaking water bath at 35 C for 30 min, and the epithelial cells were then washed free of unattached bacteria by membrane filtration as described above. Direct smears were prepared from each epithelial cell suspension and stained for 15 sec with Gram crystal violet. The number of bacteria attached to the epithelial cells was determined by direct microscopy at $\times$ 900 magnification. Up to 200 bacteria per cell could be counted with reasonable accuracy. Cells with bacteria too numerous to count were assigned a value of 300. Considerable variation in the number of bacteria attached to individual epithelial cells was observed in any given sample. This probably reflects differences in cell surface as a result of varied exposure to the oral environment. Consequently, 50 cells were counted for each sample, and an average was obtained. Control epithelial cell suspensions incubated with buffer instead of a bacterial suspension were always included to provide data on the number of bacteria which were already attached at the time of their collection. A reaction mixture containing *S. salivarius* 9GS2, an organism found to adhere unusually well to epithelial cells, was included in some experiments for comparative purposes and to serve as a positive control.

Adherence of streptococci to tongue and cheek surfaces in vivo. Strains of *S. salivarius*, *S. sanguis*, and *S. mutans* were made resistant to 2,000 μg of streptomycin per ml to study their abilities to attach to tongue and cheek surfaces in the oral cavity. Cell suspensions of the resistant strains were prepared from Trypticase soy broth cultures and standardized on the basis of optical density. Mixtures of the three streptomycin-labeled species were prepared to contain approximately 5×10^8 colony-forming units of each species per ml. Samples of the mixture (0.4 ml) were introduced into the mouths of volunteers, who were instructed to mix it with saliva. After 5 min, part of the mixture was expectorated and collected for cultural examination. After an additional 10 min, the tongue and cheek surfaces were sampled with Calgi swabs (Colab). The swabs were dissolved in buffered hexametaphosphate solution to release the organisms. Dilutions of the original mixture, the 5-min saliva sample, and the swab suspensions were prepared and plated in duplicate on Mitis-salivarius agar (Difco) plates containing 200 μg of streptomycin per ml. After 48 hr of anaerobic incubation, the plates were examined under a dissecting microscope. The proportions of the three streptococcus species were determined on the basis of their characteristic colonial morphology on this medium (3, 22).

RESULTS

Selective adherence of S. salivarius, S. sanguis and S. mutans to epithelial cells. Preliminary experiments indicated that the number of bacteria naturally attached to epithelial cells varied greatly from site to site within the mouth. Cheek cells generally averaged around 20 bacteria per per cell, whereas cells obtained from tongue scrapings or saliva sediment usually averaged 100 or more bacteria (Table 1). This suggests that the majority of epithelial cells present in whole saliva come from the tongue. The large number of bacteria they harbor is probably a reflection of the large masses of bacteria which reside in the tongue invaginations. When strains of *S. salivarius* and *S. sanguis* were incubated for 30 min with cheek and tongue epithelial cells, a significant bacterial attachment was observed (Table 1). Since cheek epithelial cells harbored the lowest number of bacteria, they were selected for most studies. All strains of *S. salivarius* and *S. sanguis* studied were found to adhere to human cheek epithelial cells. However, strain variability within these

TABLE 1. *Adherence of Streptococcus salivarius, S. sanguis, and S. mutans to oral epithelial cells*

Organism	Avg. no. of bacteria/epithelial cell			
	Human cheek	Human tongue	Germ-free rat cheek	Hamster cheek pouch
Avg. background control	19 ± 6[a]	111 ± 17	0	64 ± 10
S. salivarius 9GS2	282 ± 6		75 ± 12	133 ± 12
S. salivarius 10G3	101 ± 13	229 ± 15	47 ± 9	
S. salivarius 1A			6 ± 2	
S. salivarius SS2	99 ± 14			
S. sanguis 34	83 ± 12	190 ± 12	7 ± 2	104 ± 12
S. sanguis A15	160 ± 17		11 ± 13	
S. sanguis H20	55 ± 9			
S. sanguis H10	120 ± 14			
S. sanguis H7P			9 ± 2	
S. mutans 6715	56 ± 13		1 ± 0.5	88 ± 12
S. mutans GS5	19 ± 3		2 ± 0.5	
S. mutans HS6	46 ± 9		<1 ± 0.5	

[a] Calculated standard error of mean.

species was observed, *S. salivarius* 9GS2 being the most adherent organism encountered. In contrast to these *Streptococcus* species, the strains of *S. mutans* tested possessed feeble or no detectable ability to attach to cheek epithelial cells (Table 1, Fig. 1). The relatively weak adherence of *S. mutans* compared to *S. salivarius* was also observed with cheek epithelial cells obtained from germ-free rats and conventional hamsters.

Some parameters affecting the attachment of bacteria to human cheek epithelial cells were studied by using *S. salivarius* 9GS2. Adherence of this organism occurred at least over a range of *p*H 5 to 8, and no distinct optimum was detected. Log-phase cells harvested after 1 hr from cultures heavily inoculated with 18-hr cells tended to adhere less than exponential (3, 6 hr) or stationary phase (18 hr) cells. The use of clarified human saliva as a suspending medium in place of buffer did not influence the relative adherence of *S. salivarius* 9GS2 or the lack of adherence of *S. mutans* 6715. *S. salivarius*, *S. sanguis*, and *S. mutans* synthesize extracellular polysaccharides consisting of either dextrans or levans, or both, when grown in the presence of sucrose (3, 6, 8). However, no significant differences were observed in the attachment of sucrose- or glucose-grown cells of *S. sanguis* 34 or *S. salivarius* 9GS2 to cheek epithelial cells. Sucrose-grown cells of *S. mutans* could not be studied because of the marked aggregation of this organism which occurs in sucrose broth (9).

Selective ability of bacteria to adhere to human cheek epithelial cells. The ability of several other bacterial species to attach to human cheek epithelial cells was determined and compared to *S. salivarius* 9GS2. As in the case of the oral *Streptococcus* species studied, organisms varied widely in their ability to adhere to epithelial cells under the conditions employed (Table 2). Significant adherence was observed with *Streptococcus* strains 26 and B1, as well as with *A. naeslundii* 1 and *Fusobacterium* F7, but not with strains of *A. viscosus*, *R. dentocariosa*, *S. faecalis*, or *E. coli*. The adherence of *Streptococcus* strain 26 and B1 is of interest. Although their taxonomic position is unclear, they resemble *S. mitior*. Organisms with similar characteristics have been found to be particularly abundant on human cheek surfaces (W. Liljemark, *unpublished data*).

Adherence of streptomycin-labeled streptococci to oral surfaces in vivo. When mixtures of streptomycin-labeled strains of *S. salivarius*, *S. sanguis*, and *S. mutans* were introduced into the mouths of three subjects studied, selective attachment of these species to oral epithelial surfaces was observed in each instance (Table 3). Both *S. salivarius* and *S. sanguis* exhibited a distinct ability to adhere to tongue and cheek surfaces, but the proportions of *S. mutans* recovered from these sites were several-fold lower than the proportions present in either the original mixture or in the saliva sampled after 5 min. Because of the short experimental period, the proportional changes observed could not be influenced significantly by bacterial growth. Similarly, selective death of *S. mutans* does not seem likely because the proportions of this organism in saliva samples incubated in vitro for 15 min were not markedly altered. Thus, the oral streptococci studied exhibit selective abilities to adhere to epithelial surfaces in vivo in the mouth.

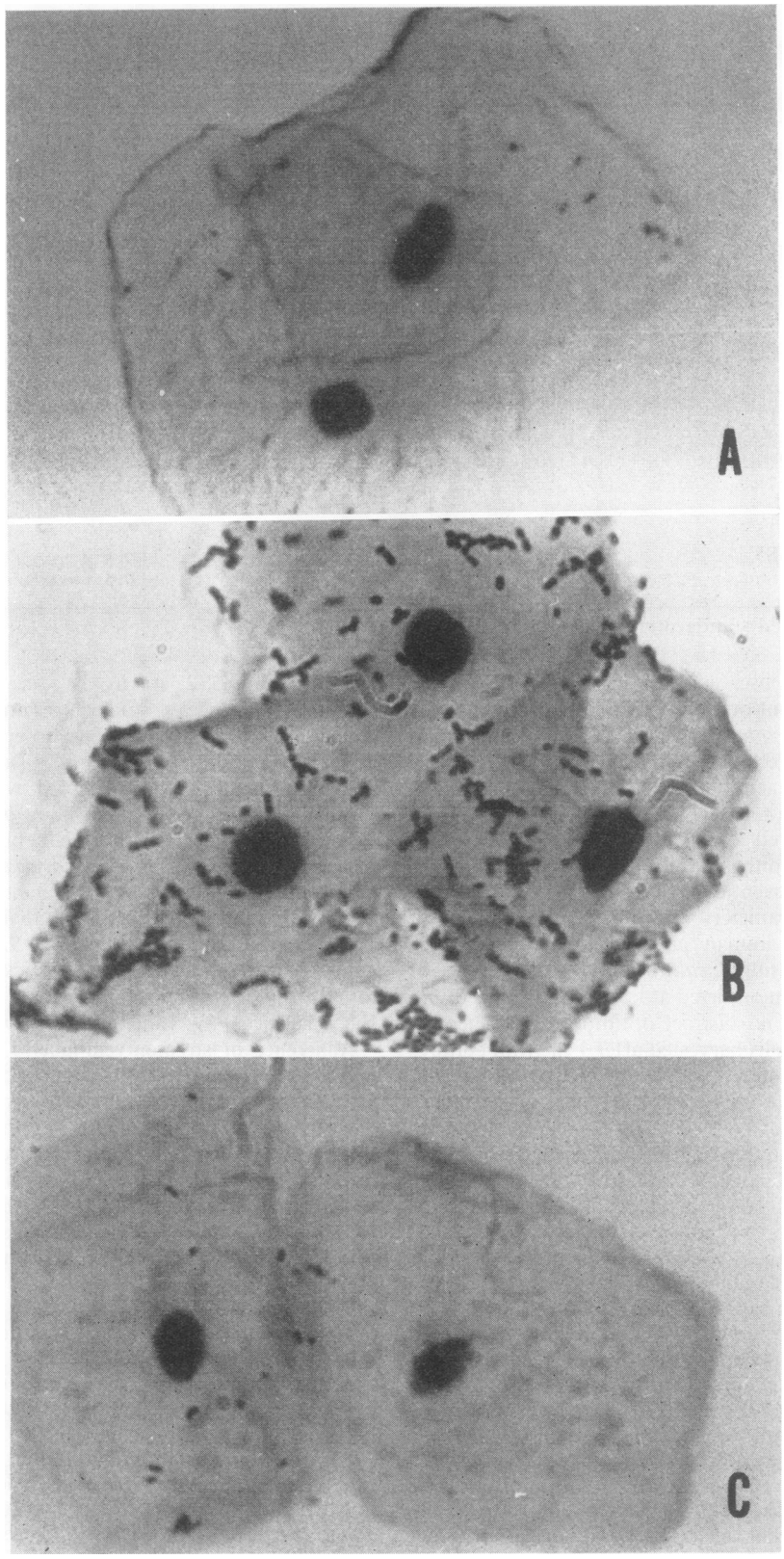

FIG. 1. *Bacteria adherent to washed human cheek epithelial cells after incubation with (A) saline (background control), (B) S. salivarius 9GS2, (C) S. mutans 6715. Reaction mixtures (2.0 ml) contained 10^5 epithelial cells and 10^8 streptococci.*

TABLE 2. *Relative adherence of bacteria to human cheek epithelial cells*

Organism	Relative adherence[a]
Streptococcus salivarius 9GS2	100
Streptococcus strain 26	33
Streptococcus strain B1	55
Fusobacterium strain F7	13
Actinomyces naeslundii strain I	49
Neisseria strain N1	7
Actinomyces viscosus strain T6	0
Nocardia strain A	0
Rothia dentocariosa ATCC 17931	0
Streptococcus faecalis	0
Escherichia coli	0

[a] Relative adherence = 100 × average number of bacteria per cell/average number of *S. salivarius* strain 9GS2 per cell.

TABLE 3. *Adherence of streptomycin-labeled strains of Streptococcus salivarius, S. sanguis, and S. mutans to human cheek and tongue surfaces*

Subject	Organism	Per cent of labeled streptococci[a]			
		Original mixture	5-Min saliva sample	Cheek swab	Tongue swab
I	*S. salivarius* 9GS2-R	19	11	41	73
	S. sanguis H7P-R	66	62	59	27
	S. mutans 6715	15	27	<0.2	<0.4
II	*S. salivarius* 9GS2-R	21	25	28	59
	S. sanguis H7P-R	33	29	61	35
	S. mutans 6715	46	46	11	6
III	*S. salivarius* Di-R	8	30	11	48
	S. sanguis M2-R	27	31	87	46
	S. mutans 6715	65	39	2	6

[a] Percentage of each organism of the total labeled cells recovered.

DISCUSSION

It is well known that oral mucosal epithelial cells contain significant numbers of bacteria which are so firmly attached to their surface that they cannot be removed by vigorous washings (12, 21). Hoffman (12, 13) observed only a limited number of morphological types of bacteria bound to human oral epithelial cells and suggested that considerable specificity might be involved in their attachment. He was unable to detect attachment of unidentified greening streptococci to washed epithelial cells, and, because papain and heating at 65 C were partially effective in loosening some bacteria normally present on the cells, he hypothesized that the attachment might entail specific antibody (13). The data obtained in the present investigation indicate that there is indeed a high degree of specificity involved in the attachment of bacteria to oral epithelial cells. In fact, different species within the same genus vary widely in ability to attach to these cells. However, there are several reasons why it is unlikely that this specificity is due to specific antibody. Previous studies have indicated that attachment of bacteria to hard nonvital surfaces, such as tooth enamel, exhibits comparable specificity (11). Thus one can have adherence specificity without the mediation of antibody. In addition, the relative differences in adherence between strains of *S. salivarius* and *S. mutans* for human cheek epithelial cells were also evident with cheek cells obtained from germ-free rats. Since germ-free animals are unlikely to contain specific immunoglobulins against these streptococci, it is unlikely that the specificity observed was due to an immune mechanism. Rather, it would seem that the adherence of bacteria to epithelial cells is due to a sorptive interaction of a surface component of the bacterial cell with some component on the epithelial cell surface. Because the surface coatings of bacteria differ widely among species (18), differences in sorptive behavior between species are to be expected. Similarly, differences in the nature of the epithelial cell surface would also be expected to influence the sorption of various bacteria. Such differences would include the degree of keratinization of the epithelial cells or changes induced by the products and metabolites elaborated by the resident oral flora.

The present investigation has shown that *S. salivarius* and *S. sanguis*, which comprise significant proportions of the streptococcal flora naturally present on the tongue and cheek surfaces of man, possess a distinct ability to adhere to these surfaces. In contrast, *S. mutans*, which is present in only minor proportions if at all on these surfaces, possesses a feeble capacity to adhere to epithelial cells. Thus, the relative ability of these organisms to adhere to oral epithelial surfaces correlates with their natural occurrence. It is also clear that differences in bacterial adherence exist between various oral surfaces, for, although *S. salivarius* attaches well to epithelial surfaces, this organism has been previously found to have poor adherence for teeth (22; van Houte, Gibbons,

and Pulkkinen, *unpublished data*). These observations also seems to explain the low proportions of this organism found on tooth surfaces.

The relatively poor adherence of *S. mutans* for oral epithelial surfaces suggests that it must adhere to teeth to become numerically predominant in the mouth. Indeed, this organism has been shown to colonize preferentially the teeth (3, 16) if it colonizes the oral cavity at all. It has only been possible to implant *S. mutans* in the mouths of experimental animals and humans when large quantities of sucrose were ingested (15, 16). Glucose or other carbohydrates cannot be substituted for sucrose, even though they promote luxurious growth of this streptococcus. *S. mutans* has been found to synthesize dextrans and other glucans specifically from sucrose which enables it to adhere and accumulate on teeth and other hard surfaces (6, 8, 9). Thus, the requirement of sucrose for oral colonization of *S. mutans* seems to be related to the adherence of the organism to teeth, rather than to its growth.

It is remarkable that the selective nature of bacterial attachment to surfaces has not been more generally considered as a potential ecological regulator of colonization. In environments which are open systems and which contain surfaces exposed to bathing fluids, one finds the bulk of the flora and fauna adhering tenaciously to the surfaces present. A flowing stream, the oral cavity, and portions of the gastro-intestinal tract are examples of such environments. Any organism which cannot attach to the rocks or bottom of a stream bed is washed away. Organisms which cannot attach either to teeth or to an oral epithelial surface are swallowed via saliva, in the case of the oral cavity, and cannot therefore colonize the mouth. A similar situation would seem to exist in the small intestine, where fluid flow and peristaltic movement have been considered to be major factors limiting microbial colonization (4). Microbial attachment to a surface most likely must occur before growth becomes important in the colonization of such environments. A number of investigators have noted the differential attachment of enteric bacteria in the intestinal canal (1, 5, 7, 17, 19), as well as of *Mycoplasma* species to trachial cells (20). In several of these studies it has been observed that virulent enteric pathogens adhere far better to the walls of the small intestine than avirulent strains (1, 5, 17). However, the potential importance of the observed adherence for colonization of these organisms has not been considered. Rather the focus has been placed upon the possible involvement of adherence in "invasiveness," or as a means of bringing damaging microbial products in more intimate association with host cells. However, colonization of a pathogen is an obvious prerequisite for pathogenicity. Consequently, the correlation between the adherence of enteric pathogens to intestinal villi with their virulence would seem to be explainable in several instances on the basis of the adherence of virulent strains permitting a sufficient degree of colonization to initiate disease. If these concepts are correct, the adherence of organisms such as *Corynebacterium diphtheriae* or *S. pyogenes* to nasopharyngeal surfaces may also be associated with virulence.

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