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Evidence that Extracellular Components Function in Adherence of *Actinobacillus actinomycetemcomitans* to Epithelial Cells

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Extracellular microvesicles and a highly proteinaceous polymer associated with a leukotoxin-producing strain, *Actinobacillus actinomycetemcomitans* SUNY 75, were shown to increase adherence of other weakly adherent *A. actinomycetemcomitans* strains to KB epithelial cells.

Actinobacillus actinomycetemcomitans is considered an important etiologic agent in periodontal disease (2, 16, 19, 20, 23). A number of *A. actinomycetemcomitans*-associated phenotypes have been proposed as potential virulence factors (14–16, 21, 23), but the mechanism(s) used by *A. actinomycetemcomitans* to maintain itself in the oral cavity and the pathogenesis of the disease remain poorly understood. *A. actinomycetemcomitans* and certain other gram-negative oral species implicated in periodontitis, including *Porphorymonas* (*Bacteriodes*) *gingivalis*, have microvesicles associated with their surface (7, 10, 15, 22). Extracellular vesicles (ExVes) bud off from membrane vesicles, which are extrusions of the outer membrane (15). The leukotoxic activity of *A. actinomycetemcomitans* is associated with microvesicles (7, 8). ExVes of *P. gingivalis* have been shown to be associated with virulence factors (4, 5, 9). In addition, it has been reported that ExVes of *P. gingivalis* are capable of mediating adhesion and coaggregation of other oral bacteria (3–5, 9, 18). The surface of certain *A. actinomycetemcomitans* cells is also associated with an amorphous material (ExAmMat) (12, 21). Recent studies in our laboratory suggested that ExVes and polymers (such as ExAmMat) associated with *A. actinomycetemcomitans* might be functioning in adherence to epithelial cells. The present study was undertaken to gain further insight into the potential of these extracellular components to modulate adherence of *A. actinomycetemcomitans*.

The *A. actinomycetemcomitans* strains used in this study were SUNY 465; SUNY 75(S), a leukotoxic strain; and strain 652, which is nonleukotoxic (7, 13, 17, 24). Bacteria were grown in Trypticase soy broth or agar containing 0.6% yeast extract (Difco Laboratories, Detroit, Mich.) at 37°C in an atmosphere of 10% CO_2 in air. For assays, bacteria from overnight cultures were diluted in fresh broth and incubated until an optical density at 495 nm of 0.2 was reached. All strains were smooth-type variants under the growth conditions used, but they exhibited distinct surface characteristics. SUNY 75(S) was enmeshed in ExAmMat, SUNY 465 was associated with ExVes, and strain 652 had little or no surface-associated material (12). The adherence assay which utilized the human oral KB cell line was a modification of an invasion assay used previously (14). Modifications to quantify adhesion included the use of confluent monolayers (6×10^5 KB cells) and 100 bacteria per mammalian cell in a 2-h assay. Moreover, bacteria were not centrifuged onto the monolayer and the gentamicin step was omitted.

Adherence levels are shown in Fig. 1. SUNY 75(S), associated with ExAmMat, adhered threefold better than either SUNY 465 or strain 652, neither of which exhibit ExAmMat. *A. actinomycetemcomitans* cultures (10 ml, approximately 10^8 organisms per ml) were washed twice with 10 ml of phosphate-buffered saline (PBS) prior to use in the assay. Adherence of SUNY 75(S) was reduced by more than 50% after washing with PBS (Fig. 1). By contrast, adherence levels of SUNY 465 and strain 652 were not affected by washing with PBS (Fig. 1). Aliquots plated prior to use in adherence assays revealed that cells were not being lost during washing and centrifugation. The first PBS washes were filter sterilized (0.20-μm-pore-size filter) to remove any whole bacteria, and aliquots were added to assay mixtures. The addition of 0.5 ml of SUNY 75(S) PBS wash to the adherence assay resulted in increased adherence by all three strains (Fig. 1). PBS washes of either SUNY 465 or strain 652 did not result in increased adherence (data not shown). The SUNY 75(S) wash caused a dose-related increase in adherence of SUNY 465 and strain 652 over a range of 0.5 to 1.5 ml (5 to 15 μg of protein) (Fig. 1). However, if either KB cells or bacteria were treated with SUNY 75(S) wash and then the KB monolayer was washed to remove unbound components, no increase in adherence was observed for any of the strains (data not shown). The SUNY 75(S) wash was analyzed for protein by the method of Lowry et al. (11) with bovine serum albumin as a standard, and total carbohydrate was assayed by the anthrone method (1) with glucose as a standard. Although the SUNY 75(S) wash (hereafter termed ExAmMat) contained 10 μg of protein per ml (100 μg per 10^9 bacteria), no carbohydrate was detected. These results indicate that SUNY 75(S) ExAmMat, which was readily removed by washing, is capable of interacting with other *A. actinomycetemcomitans* strains and promoting their adherence to epithelial cells. The results also suggest that strong binding of ExAmMat to either *A. actinomycetemcomitans* or epithelial cells occurs only when the ExAmMat is associated with both entities in the adherence interaction.

Both SUNY 75(S) and SUNY 465 (which exhibits no ExAmMat) were treated with enzymes prior to use in the adherence assay. Bacteria were suspended in PBS and treated with trypsin (1 mg/ml of PBS) (Sigma Chemical Co., St. Louis, Mo.) at 37°C. Soybean trypsin inhibitor (0.26 mg/ml of PBS) (Sigma) was added to inhibit the reaction, and the bacterial suspension was diluted further in PBS and used

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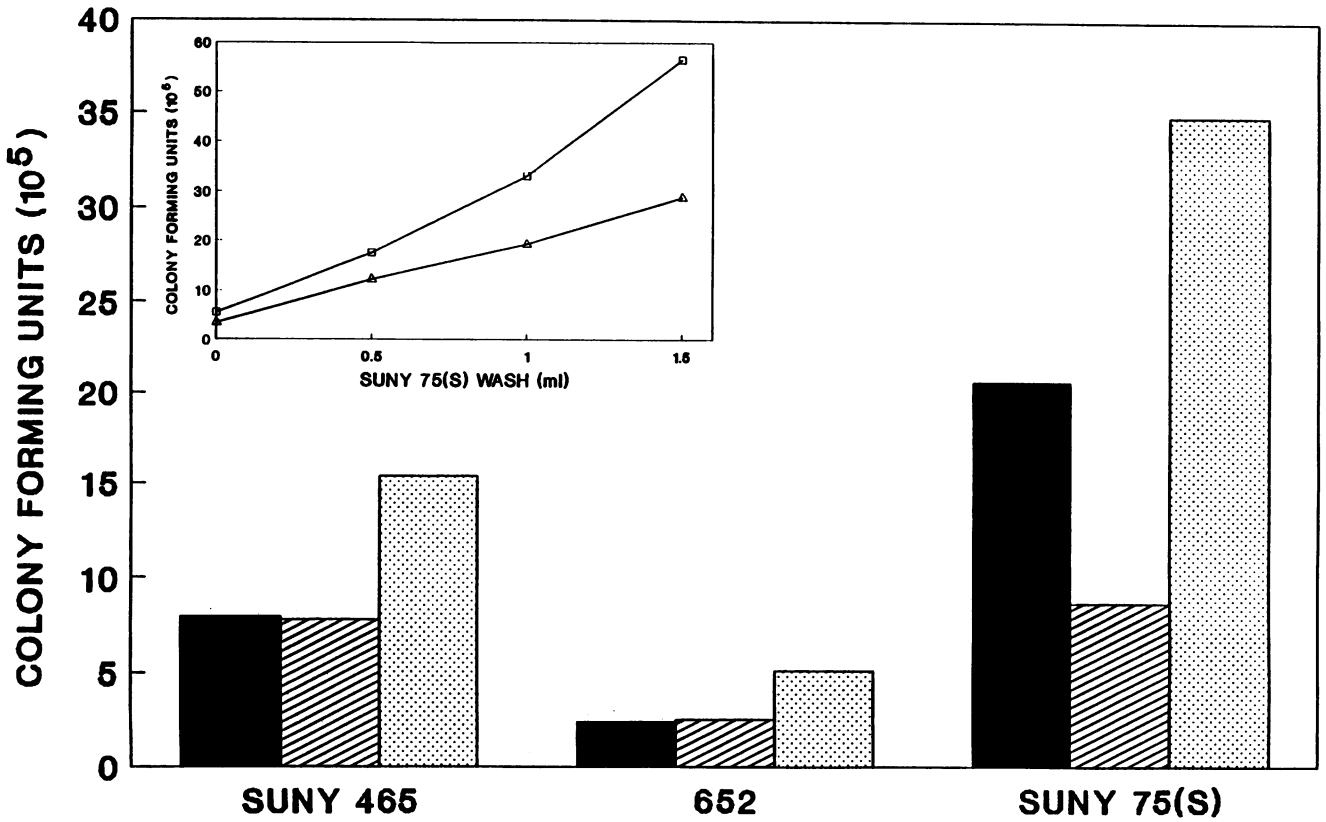

FIG. 1. Effect of SUNY 75(S) washing and washes on *A. actinomycetemcomitans* adherence to KB monolayers. Symbols: ■, control (no wash); ▨, bacteria washed with PBS prior to assay; ▩, SUNY 75(S) wash (0.5 ml) in assay milieu. One milliliter of SUNY 75(S) wash contained 10 μg of protein. (Insert) Effect of increasing amounts of SUNY 75(S) wash on SUNY 465 (□) and strain 652 (△) adherence to KB epithelial cells. Points represent the means of quadruplicate samples from a typical experiment. The standard deviation was less than 20% of the mean in each case.

in the standard adherence assay. Bacteria with soybean trypsin inhibitor served as controls. Treatment of SUNY 75(S) for as little as 4 min resulted in a decrease in adherence of 67% (Table 1). Trypsin treatment increased the bacterial number by as much as 300%, indicating that the treatment had released organisms which had been held together in clumps. Trypsin treatment of SUNY 465 resulted in a modest increase in both adherence and the number of cells (Table 1). The differential effects of trypsin on SUNY 75(S) and SUNY 465 support electron microscopy evidence that these two strains have different surface ultrastructures. Adherence and the number of cells of both SUNY 75(S) and SUNY 465 were increased (to various degrees) following treatment with neuraminidase, phospholipase C, or lysozyme (Sigma) (data not shown). The results suggest that SUNY 75(S) ExAmMat is highly proteinaceous but that nonprotein components (perhaps components of microvesicles) also play a role in *A. actinomycetemcomitans* clumping.

TABLE 1. Effect of trypsin treatment on adherence and cell number of SUNY 465 and SUNY 75(S)[a]

Strain and length of treatment	Adherence (% of control)	No. of cells (% of control)
SUNY 465		
2 min	109 ± 3	99 ± 2
4 min	113 ± 5	105 ± 5
6 min	139 ± 9	113 ± 6
SUNY 75(S)		
2 min	43 ± 4	309 ± 15
4 min	33 ± 3	309 ± 10
6 min	36 ± 2	352 ± 11

[a] Values are the means ± standard deviations of quadruplicate samples from a typical experiment.

Conditioned medium was generated as follows. SUNY 75(S) cultures (10 ml, approximately 10^8 organisms per ml) were centrifuged to pellet the bacteria and filter sterilized (0.22-μm-pore-size filter). Fresh broth treated identically served as controls. The presence of 0.1 and 0.3 ml of SUNY 75(S)-conditioned medium in the assay milieu increased adherence of strain 652 by 3.8- and 7.0-fold, respectively. ExVes bud off from membrane vesicles and are thus a component of conditioned medium (15). Hence, we isolated SUNY 75(S) ExVes from conditioned medium to determine their effects on adherence. ExVes were isolated by a modification of the method of Grenier and Mayrand (5). Briefly, SUNY 75(S) growth medium was decanted, and $(NH_4)_2SO_4$ was added over a 1-h period to a final saturation of 40%. The salt-treated medium was centrifuged at 20,000 × *g* at 4°C for 20 min, and the pellet was washed twice (by suspension in PBS and centrifugation at 27,000 × *g* for 20 min) to dilute out the $(NH_4)_2SO_4$. The pellet which contained the ExVes was

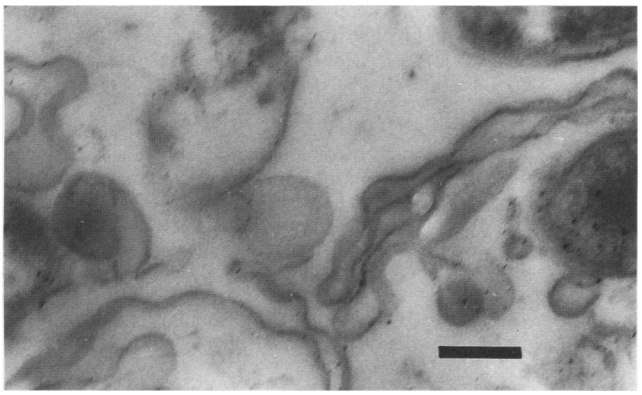

FIG. 2. Electron micrograph of SUNY 75(S) ExVes material prepared by $(NH_4)_2SO_4$ precipitation. Bar, 0.2 μm.

diluted in PBS and used immediately in adherence assays. An aliquot was also frozen and assayed later. A drop of agar was mixed with the ExVes which were prepared for transmission electron microscopy by a procedure used for whole bacteria (6), except glutaraldehyde fixation was omitted. Electron microscopy of the ExVes revealed numerous vesicles, with some contaminating whole cells (Fig. 2). Adherence of strain 652, a strain which exhibits no ExVes, was increased after the addition of SUNY 75(S) ExVes to the assay medium (Fig. 3). A maximum increase of about 450% of control (no ExVes) was observed. ExVes which had been frozen and thawed once and ExVes used directly after preparation produced similar results (Fig. 3). No viable organisms were recovered from ExVes preparations; thus, intact SUNY 75(S) in the ExVes preparation could not account for the increased CFU recovered from adherence assay mixtures containing ExVes. (*A. actinomycetemcomitans* will not survive either high salt or freeze-thawing in the absence of a cryoprotective agent.) An ExVes preparation isolated by centrifuging SUNY 75(S)-conditioned medium at 100,000 × *g* for 1 h at 4°C produced similar results (Fig. 3). The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) protein profiles of SUNY 75(S) ExAmMat and ExVes revealed the following. The most prominent bands in ExVes, 33 and 46 kDa, were not present in ExAmMat, whose most prominent bands were those at 77 and 60 (double) kDa (Fig. 4). These results indicate that after aerobic growth in broth, SUNY 75(S) is associated with both ExAmMat and ExVes and the two are most likely distinct entities.

This study suggests that both ExAmMat and ExVes enhance adherence of *A. actinomycetemcomitans*. The exact function(s) of these extracellular components in *A. actinomycetemcomitans* adherence needs to be determined. It is our hypothesis that ExVes and ExAmMat play a key role in both bacterial adherence to epithelial cells and in complex interactions between gram-positive and gram-negative oral species and the formation of dental plaque. The trypsin studies indicated that ExAmMat causes *A. actinomycetemcomitans* aggregation. That ExVes may also promote *A. actinomycetemcomitans* aggregation is suggested by the dose-response curves. Adherence levels were increased by increased amounts of ExVes, but the responses were not linear. The effect of aggregation would be to reduce the number of CFU recovered (individual organisms and aggregated organisms will both produce 1 CFU).

We have determined that SUNY 75(S) ExAmMat can interact with and increase adherence of other weakly adherent *A. actinomycetemcomitans* strains. If ExAmMat can interact in the same way with other oral species, the virulence potential of *A. actinomycetemcomitans* strains which produce the ExAmMat would most likely be increased. It could play a role in colonization of strains and species which lack potent adherence properties. The production of adhesive microvesicles by *A. actinomycetemcomitans* would

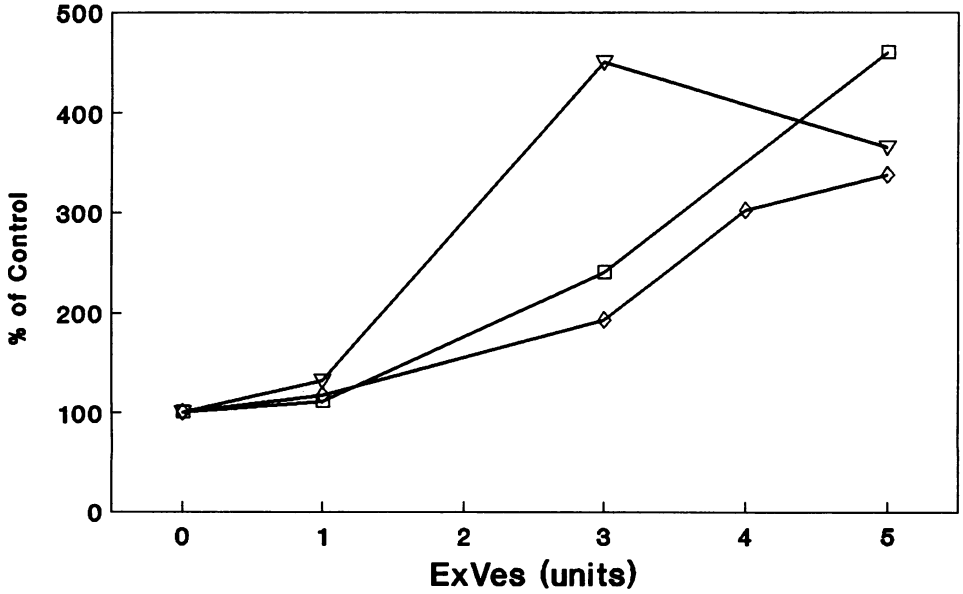

FIG. 3. The effects of increasing amounts of ExVes material on adherence of *A. actinomycetemcomitans* 652 to KB epithelial cells. One ExVes unit represents the amount of ExVes from 2 × 10^8 SUNY 75(S). ExVes were prepared by salt precipitation with (□) and without (△) freezing or by ultracentrifugation (◇). Points represent the means of triplicate samples. The standard deviation was less than 25% of the mean in each case.

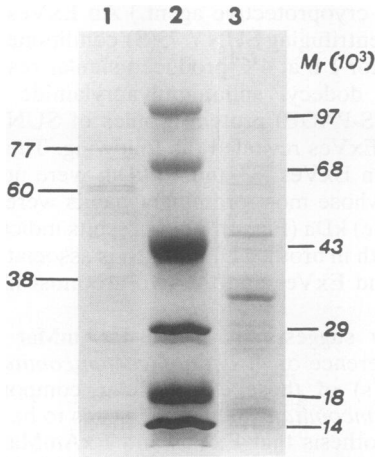

FIG. 4. SDS-PAGE analyses of SUNY 75(S) ExVes [$(NH_4)_2SO_4$ preparation) and SUNY 75(S) ExAmMat. Lanes: 1, SUNY 75(S) ExAmMat (PBS wash); 2, molecular weight standards; 3, SUNY 75(S) ExVes preparation. The separation was carried out on a 5 to 15% reducing gel by standard procedures.

most likely also increase its virulence potential. Not only could they more precisely deliver associated toxins, but also other oral species which bind *A. actinomycetemcomitans* vesicles could function as vehicles for delivery of *A. actinomycetemcomitans* toxic material. Microvesicles with adhesive properties would go a long way toward assuring the existence of *A. actinomycetemcomitans* in the oral cavity.

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