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Bacteroides gingivalis Vesicles Bind to and Aggregate *Actinomyces viscosus*

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Isolated *Bacteroides gingivalis* 2561 vesicles aggregated suspensions of *Actinomyces viscosus* and *Actinomyces naeslundii* of all taxonomy clusters. Vesicles bound near *A. viscosus* cell walls and among its surface fibrils. Tritiated vesicles bound slightly better to saliva-coated hydroxyapatite (SHA) than to SHA coated with *A. viscosus*; saturation was approached at the concentrations that were tested. Pretreatment of *A. viscosus*-coated SHA with vesicles impaired the subsequent adherence of *B. gingivalis* whole cells.

Adherence to gram-positive bacteria established within dental plaque likely favors the initial colonization of teeth by *Bacteroides gingivalis* (8), a virulent gram-negative species that has been implicated in active periodontitis. Using an in vitro system in which saliva-coated hydroxyapatite (SHA) or SHA coated with *Actinomyces viscosus* (actinobeads) was exposed to ^{3}H-labeled *B. gingivalis* cells, we have confirmed that *B. gingivalis* adheres more readily to the actinobeads and have shown that its adherence is saturable and, under saturating conditions, essentially irreversible (7). The nature of the adhesins mediating the adherence of *B. gingivalis* to actinobeads has yet to be defined (2), although surface-exposed fimbriae, lipopolysaccharide, hemagglutinin, or other outer membrane proteins of *B. gingivalis* would be in a position to interact with mutually attractive molecules on the *A. viscosus* surface.

B. gingivalis also sheds vesicles (blebs) from its surface. Grenier and Mayrand (5) have recently shown that *B. gingivalis* vesicles coaggregate strains of *Eubacterium* and *Capnocytophaga*, which do not usually coaggregate. Vesicle membranes are derived from the outer membrane of whole cells and thus contain lipopolysaccharide and outer membrane proteins. In addition to functioning in proteolysis (D. Grenier and B. McBride, Proc. Int. Assoc. Dent. Res., abstr. no. 2045, 1988), vesicles might constitute an extracellular adherence "organelle" by exposing adhesins over an increased surface area. The purpose of this study was to determine whether vesicles isolated from *B. gingivalis* bind to and aggregate *A. viscosus* cells.

Vesicles were isolated from *B. gingivalis* 2561, the standard strain used in our previous binding studies, by the method of Grenier and Mayrand (5). Briefly, bacteria were grown anaerobically for 2 days in batch cultures in Trypticase (BBL Microbiology Systems, Cockeysville, Md.) yeast extract medium containing 10 μg of hemin per ml and 1 μg of menadione per ml. The cells were removed by centrifugation, and the supernatant was brought to 40% saturation with $(NH_4)_2SO_4$. The vesicle preparation was pelleted by centrifugation at 20,000 × *g* for 40 min. It was washed and then dialyzed against 50 mM Tris buffer (pH 9.5) containing 0.5 mM dithiothreitol. The vesicles were washed in Tris buffer and centrifuged at 27,000 × *g* for 40 min. They were stored frozen in Tris buffer (pH 7.2) until use. Thawed vesicles were not refrozen, as we noted that repeated freeze-thawing diminished adherence. Transmission electron microscopy of ultrathin sections confirmed the isolation of typical vesicles (Fig. 1A). Estimates of wet weights were made from vesicle suspensions with various optical densities.

The ability of vesicle suspensions to aggregate *A. viscosus* and *Actinomyces naeslundii* strains representing the numerical taxonomy clusters of Fillery et al. (3) was tested. Washed cell suspensions were prepared in either Tris or 0.01 M phosphate buffer (pH 7.2) and adjusted to an optical density of 1.0 at 550 nm in a spectrophotometer (model 35; G. K. Turner Associates, Palo Alto, Calif.). One drop was mixed on a flocculation slide with one drop of a vesicle suspension which was diluted 1/16 from an original optical density of 1.0 (approx. 11.6 mg of vesicles per ml) and rotated at 90 rpm for 5 min. The degree of aggregation was scored visually from 0 to a maximum of 8, corresponding to a scale that was used previously in hemagglutination studies (1). For control slides, one drop of buffer was substituted for the vesicle suspension. The results summarized in Table 1 confirmed the hypothesis that *B. gingivalis* vesicles aggregate *A. viscosus* cells. With the exception of one strain, all strains of all clusters were strongly aggregated by the vesicles but not the buffer. Aggregation scores in Tris and phosphate buffer were equivalent. By electron microscopy, vesicles were observed to be bound directly to the *A. viscosus* cell surface and within the area occupied by the long surface fibrils of *A. viscosus* (Fig. 1B).

To quantify the degree of binding, *B. gingivalis* vesicles were labeled by a modification of the tritium borohydride method of Gahmberg and Andersson (4). Frozen vesicles from several purification runs were thawed, pooled, and washed twice in phosphate buffer (pH 7.4) and diluted to an optical density at 550 nm of 0.7 (approx. 9.0 mg/ml). A total of 500 μl was added to each of six conical microtest tubes, centrifuged at 27,000 × *g* for 30 min, and suspended in 200 μl of 10 mM sodium *meta*-periodate in phosphate buffer. Preliminary experiments established 10 mM as the minimum concentration yielding the highest specific activity. The tubes were kept on ice for 20 min, and the reaction was stopped by adding 20 μl of 0.1 M glycerol. The vesicles were washed twice and suspended in phosphate buffer containing ^{3}H-labeled borohydride (359 mCi/mmol; Amersham Canada Ltd., Oakville, Ontario, Canada) at 30 μCi per tube. After 30 min at room temperature, the vesicles were washed twice and suspended in 500 μl of phosphate buffer. The specific activity approximated 1.2 × 10^5 cpm/mg of vesicles. The counts per minute in the supernatant after washing totaled less than 5% of the counts per minute in the vesicle fraction.

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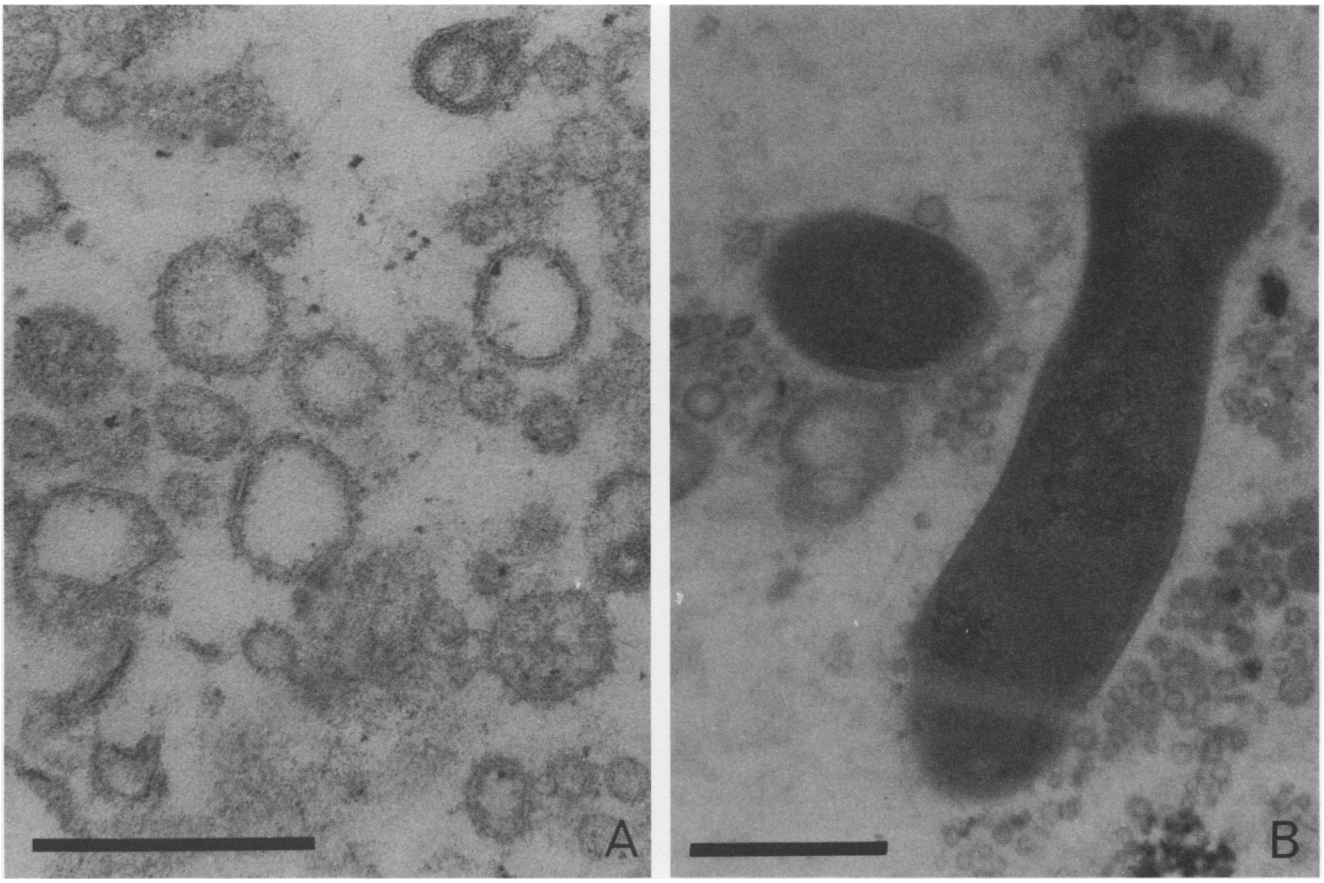

FIG. 1. Transmission electron photomicrograph of uranyl acetate- and lead citrate-stained ultrathin sections of isolated *B. gingivalis* 2561 vesicles (A; bar, 250 nm) and *A. viscosus* WVU627 cells after exposure to vesicles (B; bar, 500 nm).

Vesicles still aggregated *A. viscosus* after they were labeled with tritium.

Binding experiments with SHA and actinobeads were conducted over a wide range of vesicle concentrations, as has been done previously with whole *B. gingivalis* cells (7). Typical binding curves showed linearity over the low concentrations (Fig. 2), and saturation was approached, but not always achieved, probably because the cohesive vesicles bound to one another strongly (Fig. 1). For most runs, binding tended to be greater to SHA than to actinobeads (Fig. 2), which was the reverse situation as that described previously (7) for whole *B. gingivalis* cells. However, the vesicle-binding properties and resultant binding curves were neither as closely reproducible nor as superimposable as those for whole cells.

Inhibition experiments were performed to determine whether prior exposure to vesicles would impair the subsequent adherence of whole *B. gingivalis* cells. Actinobeads were exposed to decreasing concentrations of unlabeled vesicles in suspension or to a buffer control for 1 h, washed free of unattached vesicles, and mixed with ^{3}H-labeled *B. gingivalis* 2561 under standard saturating conditions (2.0×10^9 cells per ml for 30 min) (7). There was a clear vesicle concentration-dependent inhibitory effect on the subsequent adherence of *B. gingivalis* 2561 to the actinobeads (Fig. 3). Experiments in progress also suggest some similarities of *B. gingivalis* whole-cell and vesicle interactions with *A. viscosus*; they are both apparently heat sensitive and not in-

TABLE 1. Aggregation of *Actinomyces* strains by vesicles of *B. gingivalis* 2561

Strain[a]	*Actinomyces* species (cluster no.)	Aggregation score[b]	
		Vesicles	No vesicles
WVU627	*A. viscosus* (1)	6	0
11B2	*A. viscosus* (1)	3	0
B236	*A. viscosus* (2)	5	0
B74	*A. naeslundii* (3)	4	0
B102	*A. naeslundii* (3)	5	0
B120	*A. naeslundii* (3)	3	0
Be32	*A. viscosus* (4)	3	0
RF7	*A. viscosus* (4)	6	0
8A06	*A. viscosus* (4)	4	0
W1048	*A. naeslundii* (5)	4	0
TF11	*A. naeslundii* (5)	6	1
ATCC 12104	*A. naeslundii* (5)	3	0
398A	*A. naeslundii* (5)	3	0
WVU45	*A. naeslundii* (5)	3	0
4A05	*A. naeslundii* (5)	±	0
W1096	*A. naeslundii* (5)	5	0
B25	*A. viscosus* (6)	6	0
W1053	*A. viscosus* (6)	4	0

[a] From the culture collection of the Faculty of Dentistry, University of Toronto.

[b] Score: 0, no aggregation detected; ±, slight aggregation differing from negative control; 8, maximum aggregation yielding a single clump.

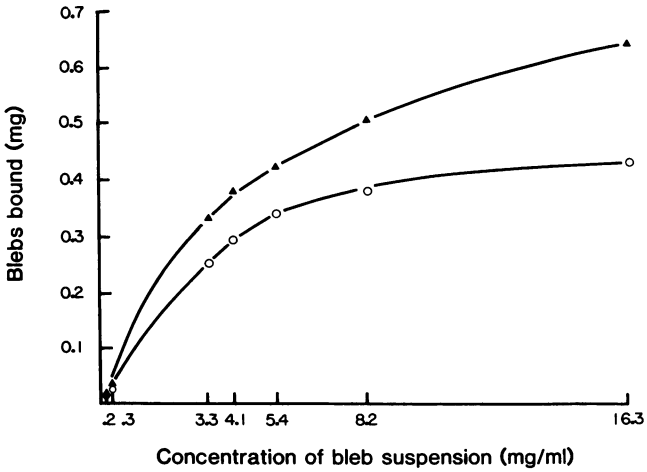

FIG. 2. Binding isotherm for ^{3}H-labeled *B. gingivalis* vesicles (blebs) bound to SHA (▲) and *A. viscosus*-coated SHA (○) over a wide range of input concentrations.

hibitable by a wide range of saccharides (2; D. Mayrand, personal communication), including *A. viscosus*-derived 6-deoxytalose (unpublished data).

Together, the data presented here support the conclusion that *B. gingivalis* vesicles both bind to and aggregate *A. viscosus* cells. Moreover, aggregation is not limited to typical *A. viscosus* and *A. naeslundii*. Therefore, it is likely that *Actinomyces* receptors for the vesicles are not uniquely associated with fibril antigens or epitopes that are not shared by the two species, for example, type 1 fibril antigen. The

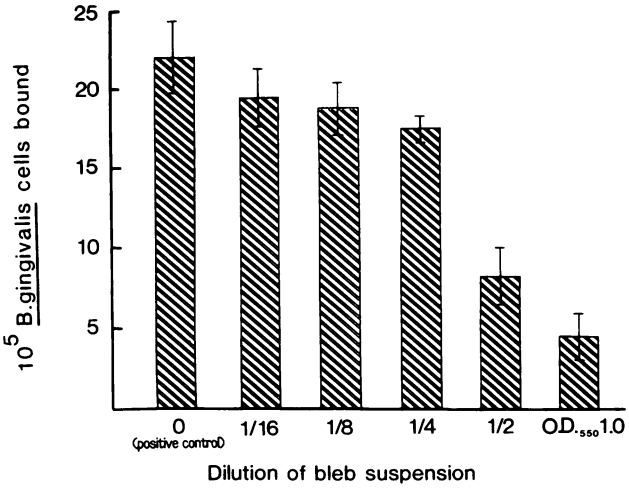

FIG. 3. Effect of pretreatment of *A. viscosus*-coated SHA with dilutions of a concentrated *B. gingivalis* vesicle (bleb) suspension on the subsequent adherence of whole *B. gingivalis* 2561 cells. OD_{550}, Optical density at 550 nm.

direct adherence of vesicles to SHA, their aggregation of several species, and their enhancement of *Streptococcus sanguis* adherence to serum-coated hydroxyapatite (U. Singh, D. Grenier, and B. C. McBride, Oral Microbiol. Immunol., in press) attest to their general adhesiveness.

Extracellular vesicles probably contribute to the virulence of *B. gingivalis* in several ways. They may act as a concentrated source of proteolytic enzymes which contribute to the ability of this asaccharolytic species to scavenge amino acids for its own survival while it degrades inflammatory mediators that are essential to host defenses (6, 9). Its toxins, including a weak endotoxin, may also be present on vesicle surfaces, and *B. gingivalis* lipopolysaccharide may influence the binding of *B. gingivalis* to *A. viscosus* (2). Host defenses may also be circumvented by the vesicles that cause coaggregation of surrounding coinfecting species (5), protecting them from factors of immunity or from administered antibiotics. While our in vitro results suggest that vesicles might also function in fostering initial contact with *A. viscosus* or other gram-positive bacteria at the tooth surface, thus increasing the probability of *B. gingivalis* establishment, such an hypothesis should be tested further in a system designed to study colonization, and not solely its cohesive component.

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