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Attachment of Oral *Cytophaga* Species to Hydroxyapatite-Containing Surfaces

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Model systems simulating the cementum portion of teeth were used to characterize the attachment process by which certain species of oral *Cytophaga* initiate the colonization of the tooth root surface in vitro. The adsorption of these bacteria to spheroidal hydroxyapatite beads and mechanically powdered root material followed Langmuir isotherm kinetics. From such data, the number of binding sites per 20 mg of substrate and the affinity constants were evaluated for two strains of *Cytophaga* sp. Resting cells of the two strains tested adhered relatively tenaciously to hydroxyapatite beads in numbers similar to those observed with cells of *Streptococcus sanguis*. Attachment of bacteria to the substrates was partially inhibited by (i) coating the substrates with human serum or saliva, (ii) pretreating cell suspensions with proteinase K or phospholipase C or D, or (iii) exposing the cells to temperatures greater than 60°C for 15 min. Treating resting cell suspensions with pronase, neuraminidase, phospholipase A2, or 0.1 M ethylenediaminetetraacetic acid had no effect on the attachment process.

A number of oral gram-positive bacteria are capable of attaching to soft (5, 7, 11) or hard (6, 11) tissues found in their environment, and many of these eventually colonize the areas proximal to their initial attachment site. Although relatively little is known of the mechanism(s) by which oral bacteria bind to hard tissues such as teeth, our knowledge of the process has been enhanced by recent studies with *Streptococcus sanguis* (1, 5), *Actinomyces viscosus* (19), and other gram-positive bacteria (3). By using particles of hydroxyapatite to simulate the surface of tooth enamel, it was demonstrated that the adsorption of oral bacteria to this material exhibited Langmuir isotherm kinetics. In addition to determining the parameters of the Langmuir equation, i.e., the maximum number of binding sites per 40 mg of substrate and the affinity constant, the studies with oral bacteria also sought to identify the environmental factors that affect adherence. For example, coating hydroxyapatite particles with salivary proteins was found to increase the adsorptive capacity of the substrate for some species of bacteria but reduce it for others (3). The effects of pH on the adsorption of certain streptococci have also been reported (12).

Whereas the literature characterizing the attachment and colonization of oral tissues by gram-positive bacteria is by no means extensive, even less is known about the colonization of these particular econiches by the gram-negative bacteria that inhabit the oral cavity. Slots and Gibbons (14) observed that *Bacteroides melaninogenicus* adsorbed to spheroidal hydroxyapatite (SHA); however, it did so in numbers 50- to 100-fold lower than *Streptococcus mutans* and *A. viscosus*. More recently, Appelbaum et al. (1) reported that the gliding bacterium *Capnocytophaga ochracea* exhibited a low K_aN value on saliva-coated hydroxyapatite, suggesting that this organism does not adhere well to that substrate.

We recently reported that several strains of gliding, non-CO_2-requiring gram-negative bacteria belonging to the genus *Cytophaga*, isolated from subgingival plaque deposits of patients with periodontitis, were capable of in vitro colonization of tooth root and exposed dentin surfaces (2). This communication describes the adsorption characteristics of two typical isolates in model systems employing SHA and powdered root material (RP) and broadly attempts to identify the outer cell membrane components involved in adsorption.

MATERIALS AND METHODS

Microorganisms. A partial description of the two strains of *Cytophaga* sp. used in this study, DR2001 and DR2002, has been previously published (2). Stock cultures were maintained in Schaedler broth (BBL Microbiology Systems) or Trypticase soy broth (BBL Microbiology Systems). After 72 h of incubation at 37°C in an atmosphere of H_2 and CO_2 (GasPak, BBL Microbiology Systems) or in air, stock cultures were maintained at ambient temperature and transferred weekly or biweekly.

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Isolation of outer membrane vesicles and membrane components. Cultures (72 h) of *Cytophaga* sp. DR2001 and DR2002 were harvested from 16 liters of Schaedler broth containing 0.5% sucrose by centrifugation in a DeLaval Cream Separator (Poughkeepsie, N.Y.). Supernatants containing outer membrane vesicles were concentrated to a final volume of 250 ml in an Amicon DC2 hollow-fiber filtration apparatus. The few remaining intact cells were removed from the concentrate by filtration through a 0.45-μm-pore-diameter filter (Millipore Corp.). The vesicles were washed once with 28 ml of 0.001 M sodium phosphate buffer, pH 6.0, containing 0.05 M KCl, 0.001 M $CaCl_2 \cdot 2H_2O$, and 0.0001 M $MgCl_2 \cdot 7H_2O$ (buffered KCl) by centrifugation in the Beckman model L350 ultracentrifuge at 88,000 × *g* for 60 min. The amber, opalescent pellet in each of the centrifuge tubes was carefully overlaid with 3 ml of buffered KCl and incubated at 4°C for 6 h to allow the upper portion of each pellet (light fraction) to resuspend slowly. The material was carefully drawn off and pooled, yielding a final volume of 36 ml. A 2-ml amount of buffered saline was used to resuspend the remainder of each pellet, and the resultant 24-ml pool was considered the heavy fraction. Sodium azide was added to the pooled suspensions to give a final concentration of 0.05%, and the materials were stored at 4°C until used.

Endotoxin preparations of both strains were made by subjecting 10 g of cell paste to the trichloroacetic acid extraction procedure of Staub (15). Lyophilized endotoxin was stored in a desiccator at −20°C. Endotoxin was also extracted from 5 g of cell paste by the phenol-water method of Westphal and Jann (18) and stored at −20°C as a lyophilized powder. Protein was determined by the biuret method (8).

Adherence assays. The assay of Clark et al. (3) was used to measure the ability of the *Cytophaga* strains to adsorb to SHA or RP. SHA of uniform size (85 to 125 μm in diameter) was purchased from BDH Biochemicals, Poole, England, and used as a substrate. A second substrate was prepared by powdering the roots of healthy, extracted, and cleaned teeth. Areas apical to the cemento-enamel juncture were fractured in a mechanical grinder; a mortar and pestle then were used to further reduce the particle size of the RP. A preparation of RP varying in size from 74 to 124 μm was obtained by sifting the pulverized material through standard 115- and 200-mesh sieves. Portions of the coarsely ground RP were deproteinized by treatment with hydrazine (16) and ground to the same size as untreated RP. Scanning electron microscopy revealed that the two materials, SHA and RP, were approximately the same size (Fig. 1); however, the latter was less spherical.

A 20-mg amount of SHA or RP was weighed out into polyethylene vials and washed several times with distilled water to remove slowly sedimenting fine particles. The substrates were then equilibrated in 2 ml of buffered KCl. Effects of normal saliva, human serum, and other biological materials on adherence were tested by incubating 20-mg samples of substrate with these substances for 12 h at 37°C on a horizontal rotary drum. The amounts of saliva, serum, or other materials used in these experiments are cited in the text. Both clarified human saliva and serum were inactivated by heating at 56°C for 30 min. After incubation, the substrate was washed twice with buffered KCl on a Vortex mixer to remove excess material.

Radiolabeled cells used in the adherence assays were cultivated in Schaedler broth containing 0.5% glucose and 0.5 μCi of [1-^{14}C]sodium acetate per ml (final concentration of 0.375 mM acetate) or Schaedler broth containing 0.5 μCi of D-[*U*-^{14}C]glucose per ml, with carrier glucose added to a final concentration of 10 mM. Cells grown in the presence of radiolabeled glucose contained twice the specific activity of acetate-labeled cells. Radiolabeled thymine and thymidine were so poorly incorporated by growing cultures of *Cytophaga* sp. when added to the medium at concentrations identical to those of glucose or acetate that a 10- to 20-fold dilution of such a cell suspension reduced the radioactivity to background levels. Cultures of DR2001 and DR2002 were harvested in the exponential phase of growth (optical density at 600 nm = 0.2 to 0.5), washed twice with buffered KCl, and resuspended to an optical density of 0.12 at 600 nm to 0.25 (20 to 40 Klett units), respectively, with the same buffer. A value of 40 Klett units was equivalent to cell densities of 6×10^8 cells and 10^9 cells of DR2002 and DR2001, respectively, per ml. These values were derived from linear slopes obtained by plotting optical densities ranging from 5 to 100 Klett units against the cell numbers estimated in a Petroff-Hauser chamber (100 fields per diluted sample). One milliliter or less of cell suspension was added to vials containing 20 mg each of SHA or RP, and the mixtures were incubated for 2 h at 37°C with gentle mixing on a horizontal drum rotating at 7 rpm; each sample was run in triplicate. After incubation, the beads were allowed to settle for 2 min, the supernatants were decanted and the SHA or RP particles were washed three times by low-speed Vortex agitation in buffered KCl. The particles of substrate and aliquots of the decanted supernatant fluid were placed in 10 ml of Insta-Gel (Packard, Downers Grove, Ill.) and counted in a Beckman LS-350 liquid scintillation counter. Quenching corrections were determined by adding a less-than-saturating standardized suspension of cells to 20 mg of SHA or RP and incubating as described above. Afterwards, the radioactivity associated with the particles, cell suspension supernatant, and three washes was determined, and the additive value was compared with that of the original cell suspension. The percent difference (5 to 15%) between the two values was used to correct for particle quenching. Binding data were also corrected for nonspecific adherence of the cells to the vial wall, generally less than 2%. Values reported in adherence assays were the average of three determinations. The maximum number of binding sites per 20 mg of substrate (N) and the affinity constant (K_a) were calculated from the direct measurements of adsorption isotherms described by the equation derived by Gibbons et al. (5), $C/Q = 1/KN + C/N$, in which C = concentration of free cells at equilibrium, N = maximum number of binding sites, and Q = cells bound to substrate. The characteristics of the Langmuir adsorption isotherm and a complete definition of the equation have been previously published (5, 10), and no further amplification is required here.

Physical and biochemical alterations of *Cytophaga* sp. cell envelopes. Resting cell suspensions were subjected to heat or enzymatic treatments to

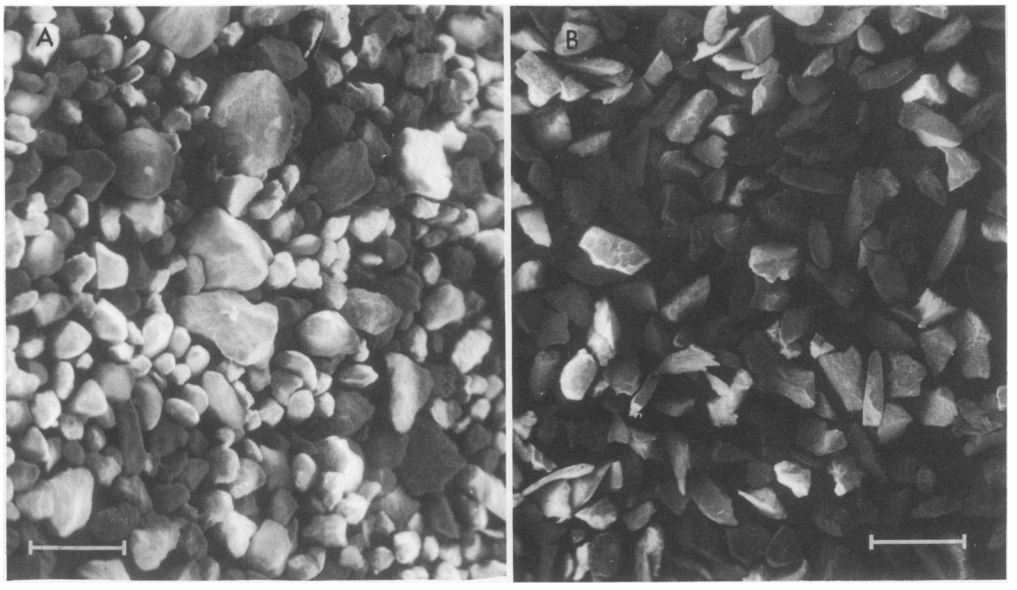

FIG. 1. *Scanning electron micrograph of the substrates. (A) SHA. (B) RP. Bar = 100 μm.*

determine which envelope components participated in the adherence process. Resting cell suspensions of strains DR2001 and DR2002 were adjusted to optical densities of 30 and 60 Klett units, respectively, and were incubated for 15 min at temperatures ranging from 50°C to boiling to determine the extent to which the attachment process was heat sensitive.

Cell suspensions adjusted to a density of 6×10^8/ml in a total volume of 3 ml were treated individually with the following commercially available enzymes for 3 h at 37°C: pronase (Calbiochem Corp.), 1 mg of protein per ml, or 135 enzyme units; proteinase K (E.M. Biochemicals), 1 mg of protein per ml, or 60 enzyme units; porcine pancreas phospholipase A_2 (Sigma Chemical Co.), 0.2 mg of protein per ml, or 340 enzyme units; bacterial phospholipase C (Sigma Chemical Co.), 3 mg of protein per ml, or 54 enzyme units; peanut phospholipase D (Sigma Chemical Co.), 0.66 mg of protein per ml, or 60 enzyme units; bacterial neuraminidase (Sigma Chemical Co.), 1 mg of protein per ml, or 0.9 enzyme units. The units of enzyme activity cited here have been defined by the manufacturer. Protease and neuraminidase treatments were carried out in buffered KCl; phospholipase A_2 treatment was carried out in 0.033 M glycylglycine buffer (pH 8.0) containing 0.03 M $CaCl_2$; phospholipase C treatment was carried out in 0.15 M triethanolamine buffer, pH 7.5, containing 0.03 M $CaCl_2$; and phospholipase D treatments were carried out in 0.075 M sodium acetate buffer, pH 5.6, containing 0.03 M $CaCl_2$. Cell suspensions were also incubated in the presence of buffered KCl supplemented with sodium ethylenediaminetetraacetate at concentrations ranging from 0.01M to 0.10M. After the 3-h incubation, the various enzymes and ethylenediaminetetraacetic acid were removed from the cell suspensions by three washes with buffered KCl, and the suspensions were readjusted to their original density. None of the above treatments altered either the size or shape of the bacterial cells, as judged by examination with a phase-contrast microscope.

Electron microscopy. Vesicular outer membrane structures were negatively stained by mixing equal parts of vesicle suspensions with a 2% solution of sodium phosphotungstate. Specimens were then layered on 300-mesh, colloidon-coated, carbon-stabilized grids, dried, and examined immediately in a Phillips 201 electron microscope with an accelerating voltage of 60 kV.

For scanning electron microscopy studies, SHA and RP containing adsorbed *Cytophaga* sp. strains DR2001 and DR2002 were washed three times with buffered KCl and fixed for 1 to 2 h in pH 6.5 Ryter-Kellenberger-buffered (9) 3% glutaraldehyde at 37°C. The particles were then washed twice in Ryter-Kellenberger buffer and fixed overnight in the same buffer containing 1% osmium tetroxide at room temperature. After the particles were washed twice in Ryter-Kellenberger buffer, particle samples were dehydrated by sequential washes through a series of 20 to 100% graded ethanol baths. Critical point drying of the samples was carried out in CO_2 with a Sorvall critical point drying system. Particle samples were then attached to stubs with double-backed tape and shadowed with gold paladium with a Technics Hummer sputter shadower. Samples were viewed with an ETEC autoscan scanning electron microscope at an accelerating voltage of 20 kV.

RESULTS

Adsorption characteristics of strains DR2001 and DR2002. In developing a model system to simulate the adsorption of *Cytophaga* sp. strains DR2001 and DR2002 to the cementum surface of the tooth root, three substrates, SHA, RP, and hydrazine-treated (deproteinized) RP, were examined. The kinetics of attachment,

as expressed by the parameters of cells free at equilibrium and cells bound, are shown for strain DR2001 and SHA in Fig. 2. Attachment is a curvilinear function typical of saturation curves that follow the law of mass action. Replotting the data according to the Langmuir adsorption model, C/Q versus Q, yielded a linear function which appears to fulfill the requirements of the isotherm equation, $C/Q = 1/KN + C/N$ (see inset, Fig. 2). Plotting the number of cells bound as a function of the total number of cells added, a linear proportionality between the two parameters was observed until cell saturation was reached (2.9×10^7 cells per mg of SHA, data not shown). In a series of experiments similar to that depicted in Fig. 2, the adherence properties of both bacterial strains were compared on the three substrates. Maximum numbers of binding sites and the affinity constants were determined from linear regression analyses of the points on the slope; correlation coefficients were also determined from these points. From data summarized in Table 1, it can be seen that the adsorptive capacity of SHA, as expressed by the N value, is greater than that of the natural substrate, RP. However, deproteinization of RP increased the number of binding sites per unit particle weight to values about the same as those observed with SHA. The affinity constants (K_a) for both SHA and RP are similar, suggesting that with either organism there is probably little difference in the adsorptive properties of the two substrates. From the correlation coefficients presented in Table 1, it can also be seen that the data do, in fact, fit the isotherm model.

Appelbaum et al. (1) observed that the magnitude of the N value derived from experiments in which *S. sanguis* was absorbed to saliva-coated SHA was dependent upon the segment

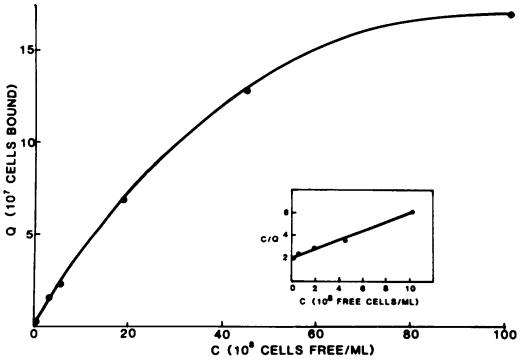

FIG. 2. *Attachment of Cytophaga sp. DR2001 to SHA beads. Assays contained 20 mg of SHA. Inset: Langmuir isotherm plot where C = concentration of cells at equilibrium and Q = number of cells bound to SHA after three washes. Each point represents average of three determinations.*

TABLE 1. *Adsorption parameters for Cytophaga sp. strains DR2001 and DR2002 interacting with three substrates*

Organism and substrate	Maximum no. of absorption sites (N)[a]	Affinity constant (K_a)[b]	Correlation coefficient
Cytophaga sp. DR2001			
SHA	2.71×10^8	1.7×10^{-9}	0.98
SHA-Saliva	1.7×10^8	3.7×10^{-9}	0.85
SHA-Serum	1.5×10^8	2.2×10^{-9}	0.83
RP	6.1×10^7	2.5×10^{-9}	0.99
Hydrazine-treated RP	1.2×10^8	3.9×10^{-9}	0.98
Cytophaga sp. DR2002			
SHA	2.4×10^8	1.5×10^{-9}	0.99
SHA-Saliva	4.0×10^7	2.0×10^{-9}	0.96
SHA-Serum	1.1×10^8	2.4×10^{-9}	0.96
RP	1.66×10^8	1.5×10^{-9}	0.98
Hydrazine-treated RP	2.3×10^8	2.3×10^{-9}	0.98

[a] All values cited were derived from experimental curves consisting of, at least, five varying cell concentrations with at least four individual samples per cell concentration. Units are cells per 20 mg of substrate.

[b] Units are milliliters per cell.

of the Langmuir plot used in the calculation. Using a series of increasing cell densities that represented far less-than-saturating cell density, these authors obtained a markedly lower N value than that derived from points taken at or near saturating levels of cells. If the lowermost three points of the slope shown in Fig. 2 are used to determine N, a value of 5.5×10^7 is obtained, whereas a calculation with the three uppermost points in the slope yielded a value of 3×10^8. Replotting data for the adsorption of strain DR2002 to SHA produced similar shifts in the value of N. Therefore, in all of the ensuing experiments, saturating or slightly less-than-saturating concentrations of cells were used in the adherence assays to appropriately measure binding.

It should also be pointed out that substituting stationary-phase cells (48-h cultures) for exponential-phase cells did not alter the N or K_a values. Scanning electron microscopy of washed SHA beads and RP particles incubated with saturating levels of DR2001 or DR2002 revealed that cells attached only to the substrate (Fig. 3). No cell-to-cell binding was observed.

Effect of saliva and serum adsorption. Clark et al. (3) reported that coating SHA beads with human saliva enhanced adsorption of *S. sanguis*, *S. mitis*, and *Actinomyces viscosus* while reducing adsorption of *S. mutans*, *S. salivarius*, and *Actinomyces naeslundii*. Treating SHA with human saliva or human serum reduced the ability of strain DR2001 and DR2002 cells to adhere to the substrate (Fig. 4). Saliva decreased the maximum number of receptor areas for strains DR2001 and DR2002 by 37 and 83%, respectively, but appeared to increase the affinity of bacteria for the substrate (Table 1).

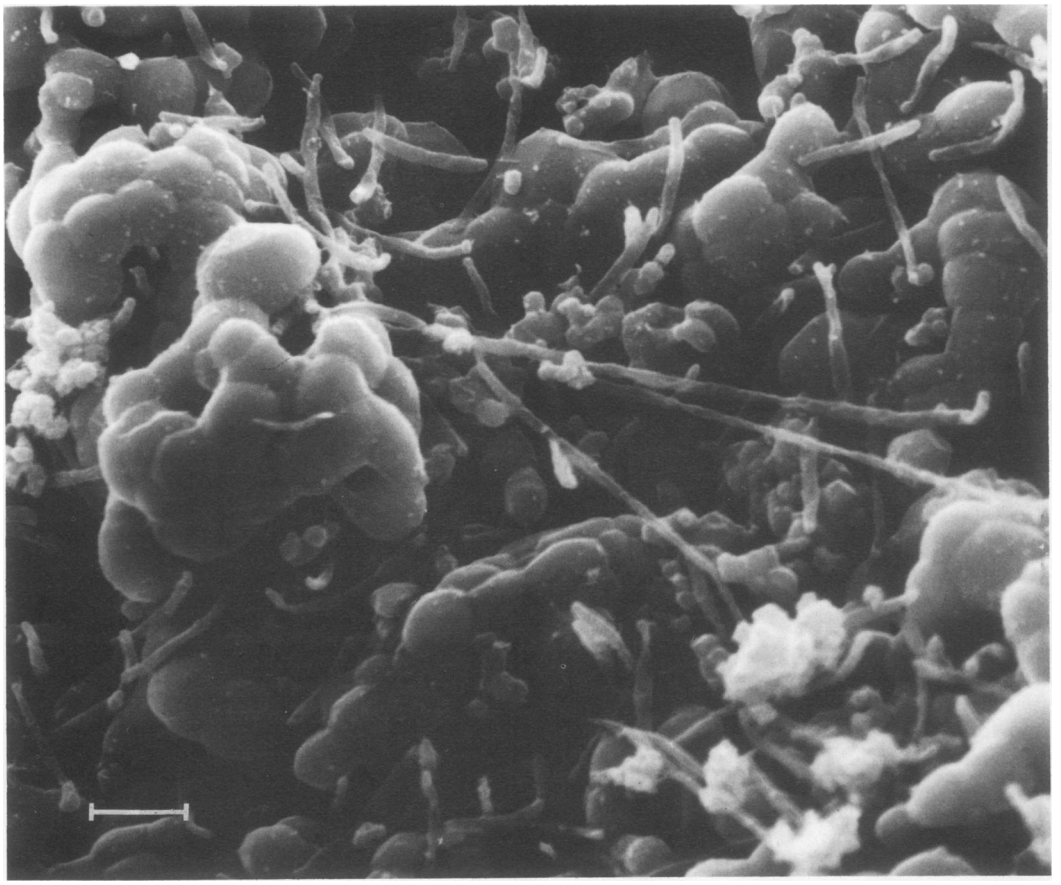

FIG. 3. *Scanning electron micrograph of a SHA bead after incubation with a saturating concentration of DR2001. Reaction mixture contained 20 mg of SHA and 10^9 DR2001 cells. Bar = 2 μm.*

Pretreating SHA with serum reduced the value of *N* by roughly 50% for both organisms. Because the results obtained with RP were very similar they are not shown. The inhibition of adsorption by saliva and serum may be relatively specific effects, since pretreating SHA and RP with equivalent amounts of two purified proteins, crystalline lysozyme and bovine serum albumin, did not affect the adsorption process (data not shown). Because the treatment of RP or SHA with saliva or serum reduced attachment of the test organisms to the respective substrates, all ensuing experiments were performed with untreated substrates to maximize the effects of attachment-inhibiting materials.

Inhibition of the attachment process by wall components. An electron microscopic examination of plaque deposits produced by strains DR2001 and DR2002 grown on root surfaces revealed that the interstices were filled with tubular and spherical vesicles that originated from the outer membranes of these organisms (Celesk and London, unpublished data). An examination of clarified culture supernatants revealed that similar structures were shed from growing cells; these vesicles were collected, separated from intact cells, and concentrated (Fig. 5). Varying concentrations of the vesicle suspension designated as the "light fraction" were incubated with SHA or RP, and the adsorptive capacity of the washed substrates was determined for both strains. Figure 6 shows that, at saturating levels of strain DR2001 vesicles (98 μg of protein per 40 mg of SHA), a decrease in cell adsorption of 89% was observed with strains DR2001 and DR2002. At low levels of vesicles, between 1 and 10 μg of protein, the inhibition of adsorption was linear and proportional to the vesicle concentration (see inset, Fig. 6). Substituting vesicles from strain DR2002 produced an essentially identical inhibition of attachment of both strains to SHA (data not shown). Treatment of RP with vesicles from either strain produced a maximum inhibition of binding of 85% at saturating levels of vesicles. Finally, no differences in the inhibition of attachment were

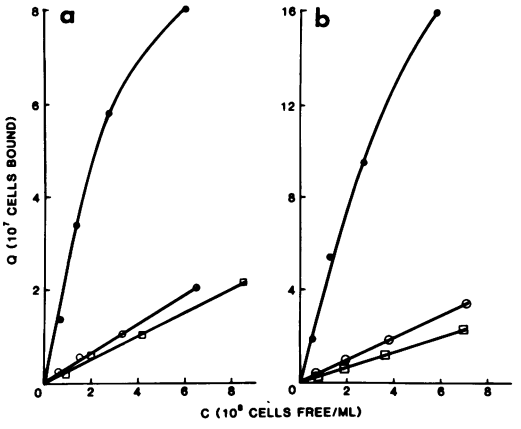

FIG. 4. *Inhibition of attachment of DR2001 and DR2002 by saliva and serum. Reaction mixture contained 20 mg of SHA preincubated with saliva (protein content = 69 mg) or human serum (protein content = 80 mg) at cell concentrations as indicated. Symbols: ●, untreated SHA; ○, saliva-treated SHA; and □, serum-treated SHA. (a) Strain DR2001; (b) strain DR2002. Each point is the average of triplicate samples.*

observed when the "heavy" vesicle fraction was substituted for the "light" fraction as long as the former was added at equivalent concentrations of protein.

From the results obtained with the vesicle preparations, it was not surprising to find that trichloroacetic acid-extracted endotoxin preparations also inhibited the adsorption of strains DR2001 and DR2002 to SHA (Table 2). However, pretreatment of SHA with phenol–water-extracted endotoxin preparations appeared to stimulate adsorption of strain DR2001 to the substrate while inhibiting attachment of DR2002. The cause of these conflicting results is not yet clear.

Effect of altering the outer membrane structure of DR2001 and DR2002 on adsorption. The preceding experiments suggested that protein and possibly lipid components of the outer membrane of *Cytophaga* sp. DR2001 and DR2002 might be responsible for their attachment to SHA. To determine more precisely those cellular components responsible for adherence, cell suspensions were exposed to heat or treated with various commercial enzyme preparations before adsorption experiments. Heating the cells at 70°C for 15 min reduced adsorption by roughly 50% (Table 3). Raising the temperature above 70°C, however, had only a minor effect on the ability of the cells to adsorb to SHA.

Pretreating cell suspensions of DR2001 or DR2002 with 0.1 M ethylenediaminetetraacetic acid, neuraminidase, pronase, or phospholipase A_2 for periods varying from 2 to 6 h had no effect on their ability to adhere to SHA. However, treatment with proteinase K, phospholipase C, and phospholipase D markedly reduced the ability of the respective strains to adhere to SHA (Table 4). Although not shown in Table 4 the effect of proteinase K could be reversed by altering the concentration of the enzyme, incubation time, or both. Lowering the concentration of proteinase K from 1 to 0.25 mg/ml or reducing the incubation time to 1 h or less consistently resulted in a 10 to 20% increase in cell adherence.

DISCUSSION

The adsorption process by which *Cytophaga* sp. strains DR2001 and DR2002 adheres to hydroxyapatite-containing substrates is similar, in at least one respect, to that observed with a number of gram-positive oral bacteria (3) in that it may be described mathematically by the Langmuir isotherm equation. By using a substrate (SHA) that was similar, if not identical, to that used by other workers (1, 3, 19), it was demonstrated that the maximum number of binding sites (N value) per unit weight of SHA for the two strains of gram-negative gliding bacteria were of the same order of magnitude as that observed with *S. sanguis* (1). Thus, in contrast to *B. melaninogenicus* (14), *Cytophaga* sp. strains DR2001 and DR2002 exhibit a decidedly greater potential for adsorption to SHA. Likewise, the affinity of the gram-negative bacteria for the substrate, as defined by the function K_a, was similar to that reported for gram-positive bacteria (1, 3). The relative strength of the cell-to-substrate interaction became apparent when it was observed that washing the cells by agitating in a Vortex mixer did not dislodge large numbers of cells. Furthermore, increasing the ionic strength of the incubation buffer 100-fold (to 0.1 M) did not appreciably reduce the number of cells adsorbed to SHA (unpublished data). It appears, therefore, that the initial attachment is not readily reversible.

Coating SHA beads with saliva or serum inhibited the adsorption of the two strains of *Cytophaga* sp. and, in this respect, the effect is similar to that observed in SHA model systems with cells of *S. mutans*, *S. salivarius*, and *A. naeslundii*. Although saliva or salivary proteins would not be expected to significantly infiltrate the plaque matrix of deep periodontal pockets, some serum components would most certainly be present in the crevicular fluid bathing the pocket. The exudate could, therefore, affect the efficacy of the adsorption process and ultimately affect colonization of the root surface in vivo. It remains to be seen whether crevicular fluid per se is capable of inhibiting adsorption of these

gram-negative bacteria to SHA or RP. In any event, pretreating SHA with whole human serum did not completely inhibit the adsorption of strains DR2001 and DR2002 to the respective substrates; roughly half of the binding sites were still available to the bacteria.

The inhibition or blocking of adsorption of *Cytophaga* cells to SHA by vesicle preparations from culture supernatant fluids suggested that the attachment of these cells to the substrate is mediated by adhesive substances already present on the outer membrane of the cell. It is highly unlikely that the vesicles synthesized an adhesive material after contact with the SHA. Furthermore, the vesicle-treated SHA beads were washed thoroughly and vigorously, so their interaction with SHA was probably of the same strength as the whole-cell interactions. The notion that adhesive ligands are integral parts of the cell membrane is supported by the fact that endotoxin preparations mimicked the blocking effect produced by vesicles. However, it must be emphasized that the endotoxin extract was a crude preparation and was probably contaminated with an array of outer membrane proteins. The actual inhibitory effect, therefore, cannot be ascribed to the endotoxin molecule per se. Little can be said at this time about the meaning of the diametrically opposed effects produced by the two lipopolysaccharide preparations. The experiments will be repeated with purified preparations of lipopolysaccharide.

The involvement of cell envelope components, specifically proteins, in the adherence phenom-

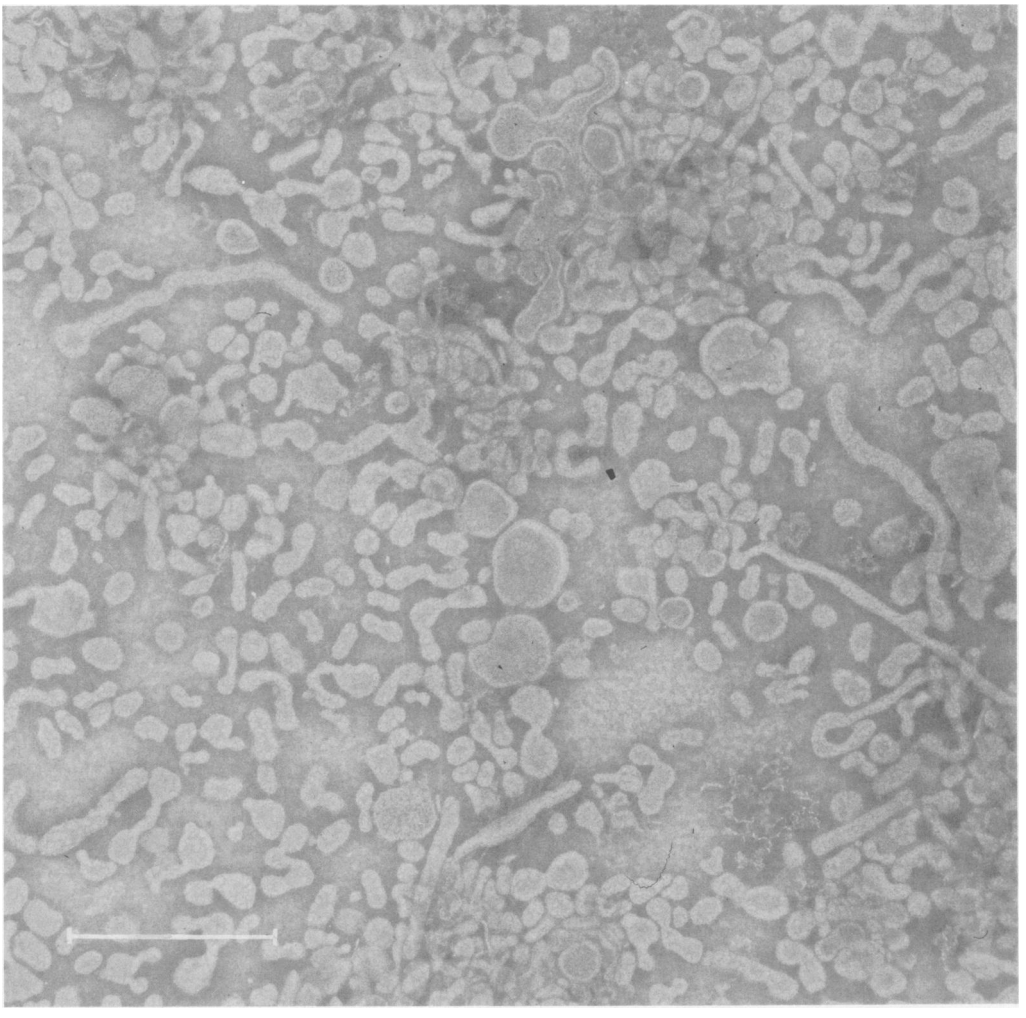

FIG. 5. *Negatively stained preparation of Cytophaga strain DR2001 vesicles. Bar = 0.5 μm.*

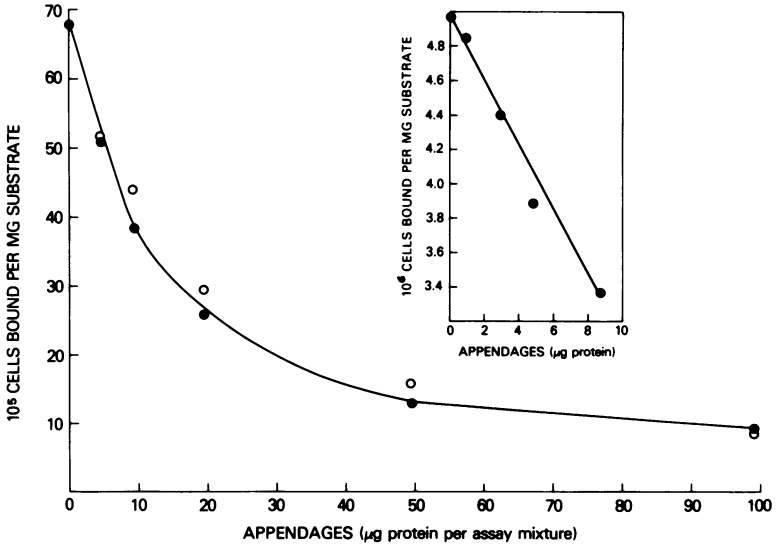

FIG. 6. *Inhibition of attachment of strain DR2001 and DR2002 cells to SHA beads by strain DR2001 vesicles (appendages). Reaction mixture contained 40 mg of SHA pretreated with vesicles and washed free of excess material and 8 × 10⁸ DR2001 cells (●) or 10⁹ DR2002 cells (○). Concentration of vesicle preparation in milligrams of protein as indicated. Inset: linear range of inhibition of attachment.*

TABLE 2. *Inhibition of attachment of Cytophaga sp. to SHA by pretreatment of substrate with lipopolysaccharide and endotoxin*

SHA treatment	Strain DR2001			Strain DR2002		
	Cells ($\times 10^8$) available	Cells ($\times 10^7$) bound[a]	% Bound	Cells ($\times 10^8$) available	Cells ($\times 10^7$) bound	% Bound
Untreated SHA	7.6	13.5 ± 0.08	18	6.8	11.1 ± 0.03	16
Phenol-water extract	7.6	17.2 ± 0.33	23 (—)[b]	6.8	6.9 ± 0.33	10 (37)
TCA extract	7.6	3.6 ± 0.61	4.7 (74)	6.8	3.9 ± 0.60	5.8 (64)

[a] Standard deviations based on four experimentally derived values. TCA, Trichloroacetic acid.
[b] Values in parentheses represent percent decrease in binding taking the control to equal 100 percent attachment. —, No inhibition.

enon can be inferred from the heat denaturation studies and protease treatments. Heating cell suspensions at 70°C for 15 min reduced their attachment capabilities by 50%. However, the failure to further inhibit the attachment process by heating the cells at higher temperatures indicates that either heat-stable proteins or nonproteinaceous substances are also involved in attachment. The degree of inhibition of attachment caused by the heat treatment correlates closely with the 50 to 55% reduction in adherence produced by exposure of the cells to proteinase K and lends support to the notion that outer membrane proteins participate in binding. Although protein components of the cell envelope are probably involved in adherence, the preceding discussion should not be construed as an argument for specific protein receptor sites as the sole mediators of attachment. The possibility also exists that heat and proteinase K treatments affected the same groups of proteins to produce conformational shifts in the outer membrane which masked the actual receptor molecules. Furthermore, evidence already in hand indicates that the attachment phenomenon is a relatively complex process involving at least one other class of cell wall components, the phospholipids.

Exposure of cell suspensions of strains DR2001 and DR2002 to the action of phospholipase C and D caused a greater inhibition of adsorption to SHA than proteinase K. Thus, the phosphatidyl glycerol moiety of lipids or lipoproteins may also play a role in the attachment process. Significantly, phospholipase A_2, which hydrolyzes the acyl group from the number two position of glycerol, had no effect on the ability of the cells to adhere to SHA. Only those phospholipases that catalyze the hydrolysis of the polar "head groups" of the phospholipid, i.e.,

TABLE 3. *Inhibition of adherence by heat treatment*[a]

Temp (°C)	DR2001			DR2002		
	Cells added ($\times 10^8$)	Cells bound ($\times 10^7$)	% Inhibition[b]	Cells added ($\times 10^8$)	Cells bound ($\times 10^7$)	% Inhibition[b]
Ambient (control)	6.8	4.9 ± 0.10	0	4.1	2.4 ± 0.5	0
50		4.9 ± 0.08	0		2.5 ± 0.05	0
60		4.8 ± 0.1	0		2.6 ± 0.7	0
70		2.9 ± 0.14	41		1.08 ± 0.3	55
80		2.0 ± 0.15	60		—	—
Boiled		—	—		1.05 ± 0.4	57

[a] Results were derived from triplicate samples used at each temperature. —, Not done.
[b] Percent inhibition calculated with control as 100% binding.

TABLE 4. *Inhibition of attachment of Cytophaga sp. strains DR2001 and DR2002 to SHA by pretreatment of cells with various enzymes*

Enzyme treatment	Strain DR2001			Strain DR2002		
	Cells ($\times 10^8$) available	Cells ($\times 10^7$) bound[a]	% Bound	Cells ($\times 10^8$) available	Cells ($\times 10^7$) bound	% Bound
Untreated control	3.0	4.5 ± 0.5	15	4.0	5.2 ± 0.3	13
Phospholipase C	3.2	1.6 ± 0.3	5 (69)[b]	4.3	1.3 ± 0.4	3 (79)
Phospholipase D	3.1	2.3 ± 0.6	7 (55)	4.2	2.0 ± 0.4	5 (63)
Proteinase K	3.2	2.6 ± 0.1	8 (50)	4.1	2.4 ± 0.3	5 (55)

[a] Standard deviation based on four experimentally derived values.
[b] Values in parentheses represent percent decrease in binding taking the untreated control to equal 100% attachment and normalizing cell input numbers.

serine, ethanolamine and choline, effectively reduced attachment of the organisms to SHA. Conversion of outer membrane phospholipid to either diglycerides or phosphatidic acid could affect the adsorption process in one of two ways. Removal of the polar groups could alter the normal interaction between phospholipid and outer membrane proteins causing regional changes in the ultrastructure or conformation of the membrane system. These changes could produce steric blocking or masking of the receptor sites. However, the action of phospholipase C and D on the outer membrane of these cells may produce a more direct effect. By separating the polar molecules from the phospholipid, the outer membrane loses cationic amino or amide groups which can interact on a strictly ionic level with the negatively charged phosphate groups in phosphate-rich regions of hydroxyapatite.

From the preliminary evidence, it appears that the attachment of *Cytophaga* sp. DR2001 and DR2002 to hydroxyapatite is a complex process involving two or more components of the outer membrane of the cells. No single enzymatic or physical treatment of cell suspensions completely obliterated the attachment process; a 70% decrease in binding was the greatest reduction effected. We are presently studying the effects of glycosidases, various sugars, and sequential enzyme treatments on the attachment process.

It has been suggested that the microbial colonization of an exposed surface in a natural environment is initiated by the reversible adsorption of one or more bacteria to that substrate (13). The weak or reversible attachment of microbe to substrate is considered to be the first stage in the adherence process and occurs when a balance between the attractive van der Waals energies and the double-layer electrical forces associated with the bacterium and substrate, respectively, is achieved. The second stage, an irreversible attachment, occurs when the adsorbed cell synthesizes an adhesive substance. The attachment of the *Cytophaga* strains to SHA may be mediated, in part, by ionic interactions between cationic amino or amide groups and anionic phosphate groups associated with the bacterium and substrate, respectively. However, the rapidity with which the interaction occurs and the strength of the attachment are better explained by postulating the presence of specific preexisting receptor molecules on the cell surface. Such an hypothesis has already been offered by Fletcher (4) to explain her data obtained with marine bacterial adherence systems.

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