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Fractionation of Hemagglutinating and Bacterial Binding Adhesins of *Bacteroides gingivalis*

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An outer membrane complex containing hemagglutinating and bacterial aggregating activity has been isolated from *Bacteroides gingivalis*. Examination of the membrane material by biochemical analysis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunological means revealed that the crude outer membrane preparation contained three major proteins and a lipopolysaccharide population that displayed size heterogeneity. At least two membrane proteins as well as the lipopolysaccharide were found to be antigenically active by immunoblot analysis. With gel chromatography and a lipopolysaccharide disaggregating buffer the membrane material was separated into two fractions. An accompanying separation of the two adherence activities was observed. The first membrane fraction, containing mostly protein and carbohydrate material, was found to contain the bacterial aggregating activity. This fraction also contained a high-molecular-weight lipopolysaccharide population. The second membrane fraction, consisting of low-molecular-weight lipopolysaccharide, protein, and loosely bound lipid was found to contain the hemagglutinating activity.

The bacterial cell surface components that mediate attachment to immobilized receptors are considered to be important pathological determinants. In the oral cavity bacteria accumulate by binding to host tissue or to other bacteria (32, 34). A number of oral organisms have been shown to possess a variety of different adhesive properties enabling them to bind to soft tissue, salivary pellicle, or other bacteria (32, 34). *Bacteroides gingivalis*, a suspected periodontal pathogen, agglutinates erythrocytes, binds to sulcular epithelium and to saliva-coated hydroxyapatite, and aggregates with a number of oral gram-positive organisms (32). Binding to saliva-coated hydroxyapatite and epithelium is inhibited by serum and crevicular fluid (32). This observation raises the possibility that the incorporation of *B. gingivalis* into dental plaque is dependent upon binding to gram-positive organisms.

The morphology of the cell surface of *B. gingivalis* has been examined in a number of studies. They have revealed a thin, electron-dense layer of capsular material staining with ruthenium red. The capsule is located external to a typical gram-negative outer membrane (17–20, 26, 35, 36); it is antigenically active and distinct from the capsular polysaccharide of other bacteroides species (17, 29). Chemical analysis of a second antigenic fraction isolated by Mansheim et al. (19) has revealed it to be a lipopolysaccharide (LPS) markedly different from that of facultative organisms (19, 20). It lacks 2-keto-3-deoxyoctulosonic acid and heptose from the core sugars and β-hydroxymyristic acid from the fatty acids present in the lipid A portion of the molecule. Fimbriae have been found on the surface of *B. gingivalis* (27, 32, 36), and a crude preparation has been found to be immunogenic in rabbits (28). Hemagglutinating activity has been suggested to reside in the fimbriae (26, 27, 32). Partially purified fimbrial preparations are hemagglutination positive. Mansheim and Kasper (18) have isolated the outer membrane components of *Bacteroides melaninogenicus* subsp. *asaccharolyticus*. The membrane was separated into two fractions, one containing the large-molecular-size polysaccharide capsule with associated proteins and lipids and the other containing the disaggregated LPS component of the membrane.

The purpose of this study is to describe the isolation and characterization of membrane components containing hemagglutinating and bacterial aggregating activity.

MATERIALS AND METHODS

Bacteria. *B. gingivalis* W12 was isolated from a periodontal lesion and identified by morphological characteristics and metabolic, and hemagglutinating activity. *Streptococcus mutans* LM7 and C6715, *Streptococcus sanguis* 10556, 10557, and 10558, *Streptococcus salivarius* HB and HBV5 (34), *Streptococcus mitior*, and *Actinomyces viscosus* were from our culture collection. All organisms were stored at −70°C in growth medium supplemented with 7% dimethyl sulfoxide. *B. gingivalis* W12 was cultured in a medium consisting of brain heart infusion (37 g), yeast extract (3 g), Trypticase peptone (10 g; BBL Microbiology Systems), hemin (5 mg), and distilled water to 1 liter. Cultures were incubated in an anaerobic chamber (Coy Manufacturing Co.) in an N_2–H_2–CO_2 (85:10:5) atmosphere. All other organisms were grown in Trypticase soy broth (BBL) supplemented with yeast extract (3 g/liter).

Isolation of outer membrane. The method used for the isolation of the outer membrane complex was a modification of the technique developed by Mansheim and Kasper (18). Cells grown for 48 h were harvested by centrifugation at 10,000 × *g* at 4°C and washed three times with 0.15 M NaCl. Organisms from 20 liters of culture were suspended in approximately 750 ml of a buffer containing 0.05 M sodium phosphate, 0.15 M sodium chloride, and 0.01 M EDTA adjusted to pH 7.4. The suspension was incubated for 30 min at room temperature, sheared by passage through a 26-gauge needle with manual pressure, and mixed in a Waring blender for 10 s. The mixture was then centrifuged at 10,000 × *g* for 20 min. The supernatant was retained and centrifuged at 80,000 × *g* for 2 h. The yellowish, gel-like, translucent pellets were suspended in distilled water, and the two centrifugations were repeated. The pellet was then again suspended in distilled water and lyophilized. This was the crude outer membrane material.

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Outer membrane material was isolated from the culture supernatants of cells grown 48 or 96 h. Whole cells were removed by centrifuging the supernatants two or three times at 10,000 × *g* to remove cells, and the supernatant was then centrifuged at 80,000 × *g* for 2 h. The pelleted material was suspended in distilled water, and the centrifugations were repeated. The pellets were then suspended in distilled water and lyophilized.

Fractionation of the outer membrane. Lyophilized outer membrane material (45 mg) was dissolved in 1.0 ml of a buffer containing 0.05 M glycine, 0.001 M EDTA, and 0.5% sodium desoxycholate (Fisher Scientific Co.) at pH 9.0. The pH was then raised to 11 to clarify the suspension and then adjusted to 9.0. The suspension remained clear when the pH was lowered, and the sample was immediately chromatographed on a 1.2- by 75-cm column containing Sephadex G100 equilibrated with 0.5% sodium desoxycholate buffer. Fractions (1 ml) were collected and monitored for protein (absorbancy at 280 nm) and for carbohydrate by the anthrone procedure.

The void volume material (pool 1) was collected and concentrated to a volume of 5.0 ml with an Amicon ultrafiltration cell (Amicon Corp., Lexington, Mass.) with a PM-30 membrane. This material was separated from the desoxycholate buffer by precipitation with 0.2 M NaCl–80% ethanol. The precipitated material was pelleted by centrifugation at 17,000 × *g* for 15 min. The pellet was dissolved in distilled water, and the precipitation and centrifugation were repeated. The remaining precipitate was dissolved in distilled water, dialyzed against distilled water for 24 h to remove excess NaCl, and lyophilized.

A second pool of material from the column (pool 2) was treated similarly to pool 1, except that a UM-05 membrane was used to concentrate the fraction.

Analytical methods. Protein was determined by Sandermann and Strominger's modification of the Lowry assay (31) with bovine serum albumin as a standard. Total sugars were measured by the phenol-sulfuric acid method (5) with glucose as a standard. Hexoses were determined by the anthrone reaction (30) with glucose as a standard. Hexosamines were determined by the method of Herbert et al. (7) with glucosamine standards, whereas muramic acid was measured by Krause and McCarty's modification of the Elson-Morgan procedure (12). The method of Kabat and Mayer (10) was used to determine methyl pentoses with rhamnose standards, whereas pentoses were determined by the orcinol method (7). Glucuronic acid was measured by the method of Blumenkrantz and Asboe-Hansen (3) with glucuronic acid as a standard. Chloroform-methanol-extractable lipid was measured by a modification of the procedure of Bligh and Dyer (2) as outlined by Mansheim and Kasper (18). Nucleic acid content was estimated by the ratio of UV light absorption at 280 nm compared with that at 260 nm (14).

Electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed with 10 or 12.5% slab gels. Gels were stained with Coomassie brilliant blue or silver stained by modification of the procedure of Oakley et al. (25). Gels were stained for LPS by the method of Tsai and Frasch (33).

For electrophoretic blot transfer of material after SDS-gel electrophoresis, the gel was sandwiched between two sheets of nitrocellulose paper that had been wetted briefly with distilled water and then soaked in 0.4% (wt/vol) SDS–1.24% Tris–5.76% glycine at 60°C for 30 min. The nitrocellulose sandwich was then placed between two stacks of 3M Whatman filter paper that had been wetted in the transfer buffer (glycine [14.4 g/liter], Tris [3.025 g/liter], methanol [200 ml/liter], pH 8.3). The entire stack was placed in the holding cassette of the Bio-Rad Trans Blot cell (Bio-Rad Laboratories), and the cell was filled with the transfer buffer. A current of 4 mA was applied overnight at room temperature. Staining of antigen-antibody complexes with antibody raised in rabbits to whole cells of *B. gingivalis* was carried out with the Bio-Rad Immun Blot (GAR-HRP) assay kit.

Bacterial aggregation assay. Bacterial aggregation by the outer membrane fractions of *B. gingivalis* W12 was measured by suspending the membrane fractions in distilled water to a concentration of 8.0 mg (dry weight) per ml.

The bacterial cells were harvested after overnight incubation and washed three times in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer (0.05 M, pH 7.2) plus 10 mM $CaCl_2$. The bacterial suspension (0.1 ml) was mixed with an equal volume of serially twofold-diluted membrane, and incubation was carried out at room temperature with shaking for 30 min. Since aggregates formed with the membranes were generally much smaller than aggregates formed with whole *B. gingivalis* cells, evaluation of the degree of aggregation was made with a Leitz stereomicroscope at ×20 magnification. The titer of the membrane activity was taken as the reciprocal of the last dilution showing aggregation of the test bacteria.

Hemagglutination assay. Hemagglutinating activity of the membrane fractions was measured by suspending the membrane material in distilled water to a concentration of 8.0 mg (dry weight) per ml. Serially twofold-diluted membrane material (0.1 ml) was mixed with an equal volume of 2.5% washed, formalinized human erythrocytes in phosphate-buffered saline (0.05 M, pH 7.2) in microtiter plates and incubated at room temperature for approximately 60 min. The titer of the membrane material was taken as the reciprocal of the last membrane dilution showing complete hemagglutination.

Serology. Antiserum was prepared in New Zealand White female rabbits by three intravenous injections of whole *B. gingivalis* W12 per week for 2 weeks. A booster injection was given in the fourth week, and antiserum was collected in the fifth week. Each injection consisted of 1.0 ml of approximately 5×10^9 O_2-killed organisms per ml in 0.15 M NaCl.

The titer of the antiserum was determined by mixing serially diluted antiserum with crude membrane (2.0 mg [dry weight] per ml) and assaying for clumping of the membranes. Antiserum was stored at −20°C.

Electron microscopy. Membrane samples were prepared for electron microscopy by a modification of the method of Kasper and Seiler (11). The crude membrane was fixed for 1 h at 4°C in 2.5% phosphate-buffered glutaraldehyde (pH 7.2). This procedure was followed by a brief wash with 1 M phosphate buffer (pH 7.2) and additional fixation for 30 min in aqueous 2% osmium tetroxide. The pellets were then dehydrated in graded solutions of ethanol up to 70%, exposed to 5% uranylacetate in 70% ethanol, and dehydrated further to 100% ethanol. The pellets were embedded in Epon Araldite for 2 days. Thin sections were stained with uranyl acetate and lead citrate and examined with a Philips 300 electron microscope.

Whole cells were prepared for negative staining as follows. Cells were fixed for 1 h at 4°C in 2.5% phosphate-buffered glutaraldehyde (pH 7.2). This was followed by two washes in phosphate buffer. A drop of the washed, suspended bacteria was placed on the top of a collodion carbon-coated grid and stained with 2% phosphotungstic acid for 30 s.

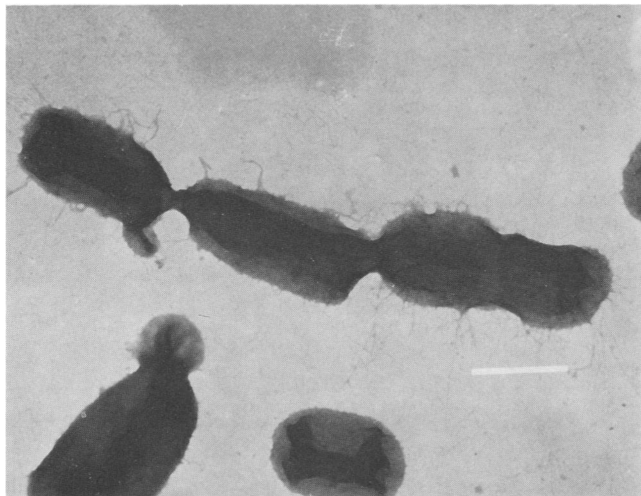

FIG. 1. Electron micrograph of negatively stained *B. gingivalis* W12. Bar, 0.5 μm.

The grid was examined with a Philips 300 electron microscope at 60 kV.

Whole cells were stained with ruthenium red by the procedure of Woo et al. (35), except that 3.0% (vol/vol) glutaraldehyde was used and fixation was carried out for 1 h at 4°C.

RESULTS

Electron microscopy. *B. gingivalis* W12 was examined by electron microscopy after staining for capsular polysaccharide with ruthenium red. The cells demonstrated typical trilaminar inner and outer membranes separated by a thin layer of peptidoglycan. A thin, electron-dense layer of capsular material located external to the outer membrane of the cell was seen in close association with the outer membrane. Fibrous and more diffuse material was seen at many locations in association with and between cells. In some cases the fibrous material extended for long distances from the cells and connected individual cells.

Whole cells were also examined by negative staining with phosphotungstic acid (Fig. 1). The cell showed a variable number of fibrillar "appendages" apparently attached to the surface of the cell. Fibrillar material was also seen in the spaces between cells apparently not attached to any particular cell. The amount of fibrillar material on any one cell was quite variable, as were the length and thickness of the individual fibers. The fibers did not appear to have a rigid structure, and some seemed to branch toward the tips.

Crude membrane material from *B. gingivalis* W12 was also examined by electron microscopy. Membrane fragments made up of single trilaminar structures were observed. Some fragments were present in long, open-ended segments, whereas other fragments had joined ends to form closed circular structures. Diffusely stained (capsular) material was also seen in the membrane preparation. Structures resembling fimbriae were never observed.

Isolation and separation of *B. gingivalis* outer membranes. One percent of the dry weight of *B. gingivalis* W12 was recovered as crude outer membrane. Further fractionation of the membranes was achieved by chromatography on a Sephadex G100 column equilibrated with 0.5% sodium desoxycholate buffer. The elution profile showed two distinct peaks of material eluting from the column (Fig. 2). Pool 1 material eluted at the void volume of the column (18). This peak contained most of the membrane protein as evidenced by the profile of optical density at 280 nm. Material was also present in a third fraction, pool 3, but the amount of material present in this fraction varied from experiment to experiment and was not studied further. Of the total material applied to the column, approximately 40% was recovered in the eluent, 45.5% was recovered as pool 1 material, and 55.5% was recovered as pool 2 material.

TABLE 1. Hemagglutinating and bacterial aggregating activities of crude and isolated membrane fractions

Membrane fraction	Hemagglutination titer (HA)	Bacterial aggregation titer (BA)	Ratio of HA/BA
Crude	1,024	512	2:1
Pool 1	2	128	1:64
Pool 2	128	2	64:1

Separation and localization of membrane activities. Throughout the isolation and separation of *B. gingivalis* outer membranes, the hemagglutinating and bacterial aggregating activities of the membranes were monitored. Crude outer membranes of *B. gingivalis* were found to have both hemagglutinating and bacterial aggregating activity. The latter included the ability to aggregate several gram-positive oral organisms, e.g., *S. mutans* LM7S and C6715, *S. mitior*, *S. sanguis* 10556, 10557, and 10558, *S. salivarius* HB and HBV5, and *A. viscosus*. *S. mitior* was chosen for further investigation. After separation of the crude outer membrane material on the Sephadex G100 column, pools 1 and 2 were tested for hemagglutinating and bacterial aggregating activity. The results are summarized in Table 1.

The ratios of hemagglutination titers to bacterial aggregation titers indicated an enrichment for bacterial aggregating

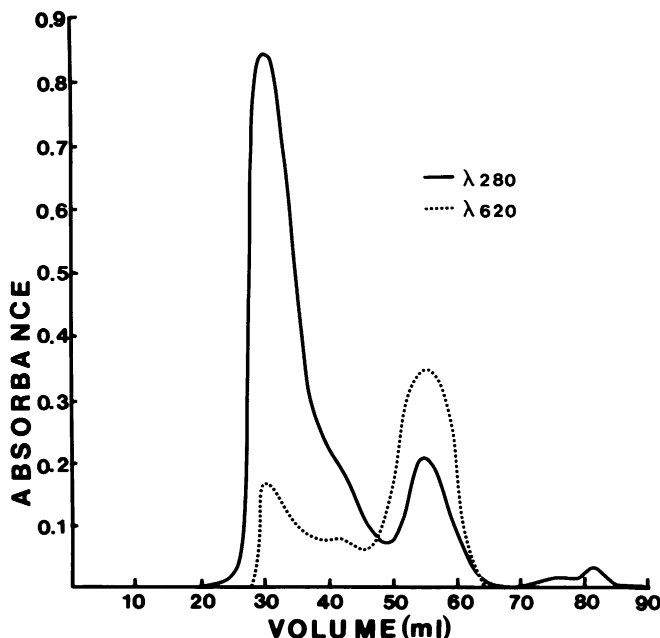

FIG. 2. Elution profile of an outer membrane preparation of *B. gingivalis* W12. Sample was applied in desoxycholate and eluted with desoxycholate. The eluent was monitored for protein (—) and reacted with anthrone reagent for carbohydrate (. . . . .).

activity relative to hemagglutinating activity in pool 1 and the reverse situation in pool 2. Thus pool 1 material appeared to contain the majority of the bacterial aggregating activity of the membrane, and pool 2 appeared to contain the majority of the membrane hemagglutinating activity. Both activities were considerably reduced after fractionation on the Sephadex column and were not recovered by combining pools 1 and 2.

Since this evidence appeared to point to these two activities being mediated by different components of the membrane, the biochemical composition of each fraction was analyzed.

Biochemical characterization of membrane fractions. The crude membrane fraction and pools 1 and 2 were characterized biochemically by colorimetric assays, and the results are summarized in Table 2. Pool 1 contained 51% protein by weight, whereas pool 2 contained 11.7% protein. In other experiments pool 2 was found to contain 5% protein; however, the separation reported in Fig. 2 involved larger volumes of material, which appeared to result in contamination of pool 2 with protein. The other principal difference was that pool 2 contained a larger amount of chloroform-methanol-extractable lipid (22% by weight) compared to pool 1 (5% by weight). Other constituents were generally equivalent.

SDS-polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was performed on the isolated membrane fractions. A 10% acrylamide gel stained for proteins with silver nitrate (Fig. 3) revealed that crude membrane material (lane B) possessed three major protein bands as well as a small number of minor bands. Pool 1 material (lane C) contained the same three protein bands as the crude membrane fraction. Pool 2 (lane D) contained one major protein band, which corresponded to the lowest-molecular-weight major protein in pool 1 and the crude membrane. Comparison of the membrane bands with standards of known molecular weight (lane A) revealed that the three major protein bands in crude and pool 1 membrane had molecular weights of 2.22×10^4, 4.15×10^4, and 6.9×10^4. One band with a molecular weight of 5.65×10^4 appeared to be more distinct in pool 1 (lane C) compared with the crude membrane (lane B).

A 12.5% acrylamide gel (Fig. 4) was used to separate the isolated membrane fractions before staining for LPS. The fractions showed regularly repeating bands throughout the length of the gel, which is characteristic of LPS preparations (33). Crude membrane (lane C) material showed up to 25 distinct bands of LPS material, a large proportion of which was at the low-molecular-weight end of the gel. Pool 1 material (lane B) showed only trace amounts of the low-molecular-weight LPS material, whereas it did contain amounts of the high-molecular-weight material roughly equivalent to that present in crude membrane. In contrast, pool 2 material (lane A) contained primarily the low-molecular-weight LPS material and only trace amounts of the high-molecular-weight LPS seen in pool 1 and crude membrane.

Immunological characterization of membrane fractions. Analysis of antigens transferred by electrophoretic blotting after SDS-polyacrylamide gel electrophoresis (Fig. 5) revealed that the crude membrane contained a number of bands that reacted with anti-*B. gingivalis* serum. The most predominant of these was a band that appeared to correspond to 4.15×10^4-dalton protein. Material in the upper third of the track stained as a dark diffuse smear, whereas the material in the lower half of the track stained in regularly repeating, diffuse bands resembling in orientation and spacing those seen in SDS-polyacrylamide gels of crude membrane stained for LPS. Pool 1 and pool 2 fractions had different staining patterns. Pool 1 material contained the diffuse, dark-staining material in the upper third of the gel as well as the 4.15×10^4-dalton protein. The regular, repeating diffuse bands in the lower half of the gel were not present in this fraction. Pool 2 material lacked darkly staining diffuse material in the upper part of the track, whereas the lower part of the track showed the repeating bands seen in the crude membrane fraction.

TABLE 2. Biochemical analysis of outer membrane fractions

Substance	Membrane fraction (% of dry weight)		
	Crude	Pool 1	Pool 2
Protein	22.1	51.1	11.7
Hexose	32.1	20.2	18.9
Total sugars	35.8	25.9	27.9
Methylpentoses	2.7	4.5	4.0
Pentose	1.6	1.3	2.1
Hexosamines	ND[a]		
Glucuronic acid	ND	ND	ND
Muramic acid	ND		
Chloroform-methanol extractable lipid	20.0	5.0	21.6
Nucleic acid	4.0		

[a] ND, Not detectable in quantities of <2 to 3%.

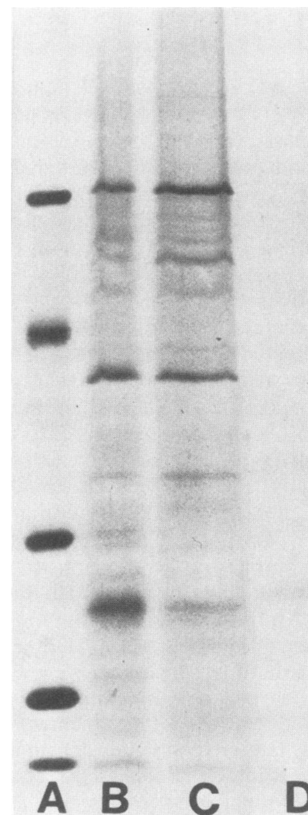

FIG. 3. SDS-polyacrylamide gel electrophoresis of crude membranes and fractions eluted from Sephadex G-100. Acrylamide concentration was 10%. Lane A contained the following molecular weight markers: bovine serum albumin (66,000), ovalbumin (45,000), trypsinogen (24,000), β-lactoglobulin (18,400), and lysozyme (14,300). Other lanes: B, crude membranes (7.0 μg of protein); C, pool 1 (7.0 μg of protein); D, pool 2 (7.0 μg of protein).

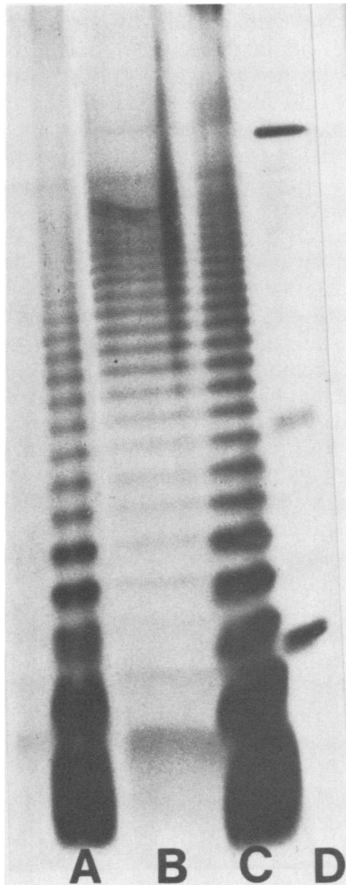

FIG. 4. SDS-polyacrylamide gel electrophoresis of crude membranes and fractions eluted from Sephaex G-100. Gels were stained for lipolysaccharide, and acrylamide concentration was 12.5%. Lanes: A, pool 2 (18 μg, dry weight); B, pool 1 (16 μg, dry weight); C, crude membranes (18 μg, dry weight); D, molecular weight markers as in Fig. 3.

DISCUSSION

Bacteria colonizing the oral cavity are often capable of binding to host tissue and to other bacteria (32, 34). In the case of *S. salivarius* it was found that the adhesins mediating binding to host tissue were distinct from those reacting with other bacteria. The fractionation of *B. gingivalis* outer membranes into two components with different binding activities demonstrates that this organism has similar characteristics. From the viewpoint of the organism this separation of adhesive constituents makes sound ecological sense. Interference with binding to one receptor does not preclude binding to an alternate receptor. *B. gingivalis* is an example of an organism that must often have to rely on binding to gram-positive bacteria because binding to the host can be inhibited by crevicular fluid or serum (32).

In experiments not reported here it was found that particulate material containing hemagglutinating and bacterial aggregating activity could be isolated from 48-h culture supernatants by centrifugation. On a dry weight basis this material contained more aggregating activity than the isolated membranes. It was thought that this material might serve as an alternate source of these membrane components; however, SDS-polyacrylamide gel electrophoresis revealed the presence of peptide bands not seen in the isolated membrane preparations which probably represent contamination due to some cell lysis. The ability of this organism to shed outer membrane fragments containing these agglutinins may serve a protective function by binding antibody that would interfere with adherence.

The separation of the hemagglutinin from the bacterial aggregating activity was not complete in the experiments reported here; however, higher resolution was achieved in experiments when small amounts of crude membrane were applied to the column. When this was done 100% separation of the activities was achieved.

Biochemical studies of the two membrane fractions revealed that they had basically the same composition as those isolated by Mansheim and Kasper (18). Pool 1 consisted of a large amount of protein and carbohydrate material with little chloroform-methanol-extractable lipid, whereas pool 2 consisted mostly of loosely-bound lipid, carbohydrate material, and a small amount of protein. The distribution of membrane material into pool 1 and pool 2 fractions after column chromatography as well as the total amounts of carbohydrate and chloroform-methanol extractable lipid present in the two pools varied significantly from the results obtained by Mansheim and Kasper (18). These differences could be due to strain variations in the composition of the outer membrane complex or to the fact that the method used here for the isolation of outer membranes was modified by omitting the heating step. It was found that heating the cells in the sodium chloride-sodium phosphate-EDTA buffer resulted in a loss of hemagglutinating and bacterial aggregating activity. Further information about the composition of the

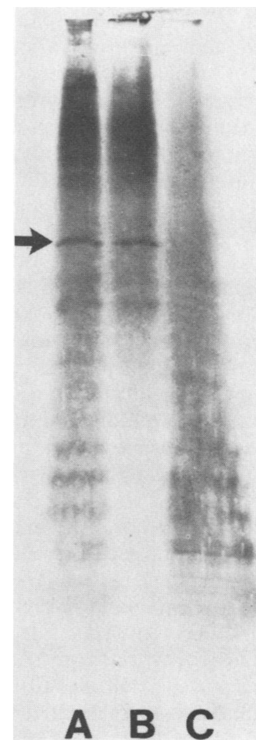

FIG. 5. Western blot of SDS-polyacrylamide gel electrophoresis of crude membranes and fractions eluted from a Sephadex G-100 column. Antigens transferred to nitrocellulose were reacted with anti-*B. gingivalis* serum. Lanes A, crude membranes, 7.0 μg of protein (32 μg, dry weight); B, pool 1, 7.0 μg of protein (14 μg, dry weight); C, pool 2, 7.0 μg of protein (63 μg, dry weight). The arrow indicates the position of the 4.15×10^4-dalton protein.

membrane fractions was obtained from SDS-polyacrylamide gel electrophoresis. The presence of LPS in crude outer membranes was demonstrated by staining with the LPS stain of Tsai and Frasch (33). The banding pattern indicated that the LPS present in the crude membrane fraction exhibited size heterogeneity. This phenomenon has been observed in several other organisms (6, 8, 13, 15), but has not been reported for *B. gingivalis*. The observation of LPS size heterogeneity has led to the proposal that smooth-type organisms contain a mixture of LPS molecules, some lacking O-antigenic side chains and others containing various numbers of covalently bound O-antigenic side chain units. Rough variants contain large amounts of the low-molecular-weight LPS and little of the high-molecular-weight material. Separation of crude membrane into pool 1 and pool 2 fractions with the LPS disaggregating buffer resulted in the fractionation of the LPS component of the membrane, with the majority of high-molecular-weight LPS remaining in pool 1 and the low-molecular-weight LPS eluting in pool 2. This is in contrast to the previously reported distribution of *B. gingivalis* LPS after disaggregation by sodium desoxycholate (18), with LPS located only in pool 2. Darveau and Hancock (4) have reported that the proportion of high- and low-molecular-weight LPS molecules varies as a function of the extraction procedure.

The protein banding patterns of crude outer membrane seen in silver-stained SDS-polyacrylamide gels are typical of outer membrane preparations from other gram-negative organisms (1, 16, 23) in that there are only a few major protein bands present; our outer membranes contained three major proteins with molecular weights of 69,000, 41,500, and 22,000. Five major peptide bands were reported to be present in the outer membrane preparation of Mansheim and Kasper (18), three of which had molecular weights in the range of those seen here. The identity of the peptide bands seen here has not been determined. However, studies with a number of enteric bacteria have shown that the outer membranes contain proteins (porins) that act to make the outer membrane selectively permeable or impermeable to certain substances (21, 22, 24). The porins vary in size from strain to strain, but are generally major outer membrane polypeptides with molecular weights of 35,000 to 40,000. One can speculate that the 41,500-dalton peptide observed in the outer membrane of this organism is a porin. The 22,000-dalton peptide has a molecular weight in a range consistent with it being a pilus or fimbria subunit (9).

Immunological studies also indicated that pools 1 and 2 are made up of different membrane components. Immunoblots demonstrated that pool 1 contained two protein bands that reacted with antibody raised to whole cells, whereas pool 2 did not appear to contain any well-defined protein antigens. Both pools contained antigenically reactive LPS; pool 1 contained primarily the high-molecular-weight forms, and pool 2 contained the low-molecular-weight LPS. The capsular polysaccharide of this organism is also antigenically active (17, 18, 29); however, because of its large molecular size, reported to be 7.2×10^5 daltons (18), it probably did not enter the gel. The fimbriae of *B. gingivalis* are also reported to be antigenically active (28); however, this observation should be viewed with caution as it was not established that the fimbrial preparations were homogenous. It is possible that other outer membrane components that are known to be antigenically active, such as LPS (19) and capsular polysaccharide (18), were present as contaminants and that the Ouchterlony reaction was detecting antibodies raised to these components. Second, although it was shown that serum from rabbits immunized with these preparations was able to interfere with hemagglutination, controls with nonimmune serum were not included. Previous studies (32) and experiments in our laboratory (manuscript in preparation) have shown that nonimmune rabbit serum alone inhibits hemagglutination. If the 22,000-dalton protein seen in gel electrophoresis (Fig. 3) is a fimbrial subunit, it was not observed to react with antiserum raised against whole cells.

Further characterization of the pool 1 and pool 2 fractions is being carried out to determine the nature of the hemagglutinating and bacterial aggregating components in these two pools. However, one can speculate on what components are involved at this point. Pool 1 is reported to be composed of a capsular polysaccharide-protein complex (18). This study has also shown that high-molecular-weight LPS is present in this fraction. Any of these components could theoretically be responsible for the bacterial aggregating activity. However, the fact that LPS is present in both pools 1 and 2, whereas the bacterial aggregating activity is virtually all in pool 1, would seem to indicate that either LPS is not involved in this activity or only high-molecular-weight type LPS is active in bacterial aggregation.

The pool 1 fraction aggregated a number of gram-positive bacteria (unpublished observation). The limited number of components available suggests that possibly the same adhesin is involved in all the interbacterial binding reactions. If this is the case, then the adhesin must recognize a receptor common to many gram-positive organisms. In light of the uniqueness of the specific chemical composition of the cell wall, it is possible that the adhesin reacts with charged groups on the cell surface.

Pool 2 contains low-molecular-weight LPS, chloroform-methanol extractable lipid, and a small amount of protein that appeared to be due primarily to the presence of the 22,000-dalton peptide. Hemagglutination has been reported to be due to the presence of fimbriae on *B. gingivalis* (26, 27, 32). It was not possible to determine whether fimbriae were responsible for the activity observed here. Negatively stained cells did show fimbria-like structures; however, membrane preparations examined by electron microscopy did not appear to contain fimbriae. Studies with whole cells (data not shown) indicated that procedures used to remove fimbriae from the cells had no effect on the hemagglutinating activity of whole cells. On the other hand, the protein present in pool 2 had a molecular weight in the range suggestive of a fimbria (9). A recent study that suggests that fimbriae are responsible for hemagglutination also indicated that LPS did not appear to be involved in hemagglutination, since phenol-water-extracted LPS did not cause hemagglutination and was unable to block the hemagglutinating activity of whole *B. gingivalis* cells (26). This would appear to implicate the lipid-protein portion of pool 2 in hemagglutination.

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