JOURNAL OF BACTERIOLOGY, May 1990, p. 2527–2534
0021-9193/90/052527-08$02.00/0
Copyright © 1990, American Society for Microbiology
Vol. 172, No. 5

Purification and Characterization of a *Bacteroides loeschei* Adhesin That Interacts with Procaryotic and Eucaryotic Cells

JACK LONDON* AND JANET ALLEN

Laboratory of Microbial Ecology, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20892

Received 20 October 1989/Accepted 15 January 1990

The adhesin of *Bacteroides loeschei* PK1295 that mediates coaggregation with *Streptococcus sanguis* 34 and hemagglutination of erythrocytes was purified to electrophoretic homogeneity. The lectinlike protein has an estimated native M_r of 450,000 and consists of six subunits of identical molecular weight (M_r 75,000). The purified adhesin appears to be a basic protein with a pI between 7.4 and 8.0. Amino acid and N-terminal sequence analyses were carried out with the purified protein. These indicated that the protein contains a large number of Asx and Glx residues as well as basic amino acid residues. The binding site of the pure adhesin retained its native configuration during purification. When preincubated with streptococcal partner cells at pH 4.6, the adhesin prevented *B. loeschei* cells from coaggregating with the streptococci. An adhesin preparation adjusted to a pH of 6.8 rapidly agglutinated both streptococci and neuraminidase-treated erythrocytes. Galactosides inhibited the agglutination reactions.

The association between fimbriae (pili) and their role in the adhesive functions of enteric bacteria (e.g., hemagglutination and attachment to eucaryotic cells) was made more than 30 years ago (6, 7). However, remarkably little is known about the physical and chemical nature of fimbria-associated bacterial adhesins, because the genes encoding the adhesive proteins were distinguished from those encoding the fimbria monomers in two large *Escherichia coli* operons only 5 years ago (19, 21). These studies established that the adhesin proteins and structural subunits of fimbriae in those two systems were distinct entities. Also, isolation of significant quantities of adhesin appears to be a problem because they apparently exist in comparatively small numbers on the surface of bacterial cells. Published reports estimate that the ratio of adhesin molecules to fimbrial monomers is somewhere between 1:60 (8) and 1:200 (9). What information is available about the structure of adhesins has been deduced from *E. coli* DNA sequence data of cloned fimbrial operons that contain the gene encoding the adhesin (16, 17) and two recent investigations which described some of the properties of P and type 1 adhesins (8, 9); the latter study included the N-terminal amino acid sequence of the type 1 adhesin. Although essentially all of the data available on fimbria-associated adhesins at present are derived from studies with enterobacteria, this situation is changing rapidly. Interest in this class of proteins has intensified in many areas of microbial pathogenesis and ecology. The human oral cavity is one such area of study; a growing number of descriptive studies indicate that adhesin-receptor interactions are common among oral bacteria and that these molecules play an important role in the colonization of oral tissues (14).

Bacteroides loeschei PK1295, a human oral isolate, synthesizes an adhesin which mediates its coaggregation with *Streptococcus sanguis* 34 (22) and hemagglutination of a variety of neuraminidase-treated mammalian erythrocytes (23). Adhesin-specific monoclonal antibodies (MAbs) prepared by immunizing mice with adhesin-bearing fimbriae were screened for inhibition of coaggregation (24) and found to be potent inhibitors of both coaggregation and hemagglutination. These MAbs were used to make a preliminary identification of the adhesin (24). Subsequently, the MAbs were used to estimate the number of adhesin molecules per cell, and immunoelectron microscopy revealed that the adhesins were associated with the distal portion of the fimbriae of the microorganism (25; J. London, unpublished data).

In this report, the anti-*B. loeschei* adhesin MAbs were used to prepare affinity matrices which provided the primary method of purifying the streptococcus-specific adhesin to electrophoretic homogeneity. We present evidence that the protein composed of the M_r 75,000 subunits is, in fact, the streptococcus-specific adhesin, and we describe some physical properties, the amino acid composition, and the N-terminal amino acid sequence of this large lectinlike protein.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *B. loeschei* PK1295 and *S. sanguis* 34 were grown in screw-cap tubes containing Schaedler broth (BBL Microbiology Systems, Cockeysville, Md.) at 37°C under anaerobic conditions. Large-scale cultures were grown in 1-liter bottles; 20 liters of medium yielded roughly 40 g (wet weight) of cells. Cell pastes were stored at −20°C until used.

Purification of the streptococcus-specific adhesin. Affinity gels were prepared by activating 1 g of CNBr-Sepharose 4B (Pharmacia LKB, Uppsala, Sweden) and reacting it with 15 to 20 mg of MAb 5BB1-2 or 3AD6 (24) as specified by the manufacturer. The washed Sepharose beads bearing the coupled antibody were stored in 200 mM sodium borate buffer (pH 8.0) containing 0.8% NaCl (BBS) at 4°C until used.

The adhesin was released from *B. loeschei* PK1295 by suspending 8 to 10 g of cells (wet weight) in 20 ml of BBS containing the protease inhibitors phenylmethylsulfonyl fluoride (5 mM) and EDTA (10 mM) and subjecting the continuously cooled cell suspension to ultrasonic disruption with a Branson model 350 sonifier (Heat Systems, Plainsview, N.Y.) operating at 70% of maximum power output for 4 min. Unbroken cells and membrane fragments were removed by centrifugation at 240,000 × *g* for 60 min in a Sorvall model

* Corresponding author.

ADT75B ultracentrifuge (Du Pont Co., Newtown, Conn.). The supernatant was concentrated to a volume of 4 to 5 ml in a filtration unit (Amicon Corp., Danvers, Mass.) equipped with a YM-10 low-protein-binding membrane, and the concentrate was added to a vial containing 300 mg of the MAb-conjugated beads. The suspension containing beads and sonic concentrate was mixed by end-over-end rotation for 14 h at 4°C. After mixing, the beads were separated from the concentrate by low-speed centrifugation (4,000 × *g* for 3 min), and the concentrate was saved for further analysis to determine whether all of the adhesin had been removed. The Sepharose beads carrying the immune complex, MAb, and adhesion were rinsed once with 3 ml of BBS containing protease inhibitors, three times with 3 ml of BBS containing protease inhibitors plus 2 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS) to remove nonspecifically adhering hydrophobic proteins, once with 3 ml of BBS containing 2 mM CHAPS plus 1 M NaCl to remove hydrophilic contaminants, twice with 3 ml of BBS containing 2 mM CHAPS, and six times with 3 ml of 20 mM Tris hydrochloride (pH 8.0) to remove any remaining contaminating adherent material. Adhesin was eluted by treating the beads three times with 3 ml of 5% acetic acid. Acetic acid washes were concentrated to a volume of 0.5 to 1 ml by Centricon (Amicon) filtration. The pH of the solution was then increased to 4.6 by several passages of the adhesin solution through the concentrating filter, with the lost volume replaced with 50 mM sodium acetate buffer (pH 4.6) containing 0.02% azide. This procedure yielded between 200 and 300 μg of adhesin protein. In some instances, the pH of the adhesin solution was raised to 6.8 by the addition of NaOH and 0.1 M Tris hydrochloride buffer (pH 6.8). The adhesin was stored at −20°C.

The efficiency of the recovery was estimated by resolving samples of the purified adhesin, supernatant fluid following adsorption to the affinity beads, and the pellet of the centrifuged ultrasonic sample on sodium dodecyl sulfate-gels, immunobloting the separated polypeptides, and developing the nitrocellulose filters with the appropriate antibody-conjugate system (see below). Scanning the intensity of the stains with a laser densitometer (XL Ultrascan; LKB, Uppsala, Sweden) and calculating the area under the peaks indicated that between 85 and 90% of the adhesin had been recovered by the MAb affinity matrix. Assuming that *B. loeschei* is similar to other gram-negative bacteria, water makes up 70% of the cell total weight, and 55% of the dry weight is protein (10). Thus, of a 10-g (wet weight) cell pellet, 1.65 g is protein. With a yield of 300 μg of adhesin protein, the adhesin represents roughly 0.02% (300 μg/[1.65 × 10^6 μg] × 100) of the total protein of the cell. The protein concentration was determined by using the commercially available protein assay kit from Bio-Rad Laboratories, Richmond, Calif.).

Preparation of rabbit polyclonal anti-adhesin antibody. After a 30-ml sample of blood had been withdrawn, a 4-month-old female New Zealand White rabbit was given three intradermal injections consisting of a total of 150 μg of the streptococcus-specific adhesin emulsified in 1 ml of adjuvant (Ribi Immunological Research, Hamilton, Mont.) over a period of 6 weeks. Blood was subsequently withdrawn from the central ear artery of the immunized rabbit and allowed to clot overnight, and the serum was stored at −20°C until used. Rabbit immunoglobulin G (IgG) was purified by a two-step procedure involving ammonium sulfate precipitation and DEAE ion-exchange chromatography (26). The IgG was ultimately made up in 0.02 M phosphate buffer (pH 7.2)–0.78% NaCl (PBS) and stored at −20°C until needed.

Coaggregation inhibition and hemagglutination studies. Quantitative coaggregation experiments were performed by using the spectrophotometric procedure of McIntire et al. (18). Inhibition of coaggregation studies with anti-streptococcus-specific adhesin serum were performed as follows. A series of test tubes containing a suspension of 10^9 *B. loeschei* cells in coaggregation buffer (CAB; 0.001 M Tris hydrochloride [pH 7.4], 0.15 M NaCl, 0.0001 M $CaCl_2$, 0.0001 M $MgCl_2$, 0.002% NaN_3) received anti-adhesin IgG (in PBS) in the range of 98 to 200 μg of protein to a volume of 400 μl. The mixture was incubated at room temperature with constant shaking for 30 min. After the incubation period, 200 μl of a suspension of *S. sanguis* 34 (10^9 cells) was added to each tube to a final volume of 600 μl, the contents of each tube were mixed vigorously for 5 min, and the tubes were centrifuged for 1 min at 500 × *g*. The supernatants were diluted 1:2 with CAB, and the optical density at 600 nm was measured with a spectrophotometer (model 2400; Gilford Instrument Laboratories, Inc., Oberlin, Ohio). Controls consisted of mixtures of both suspensions containing PBS instead of IgG or 200-μl suspensions of each organism brought to a volume of 600 μl with CAB. The percent inhibition of coaggregation was determined as previously described (18).

Qualitative studies with the purified adhesin as the inhibitor were performed as follows. A suspension of 2.5 × 10^8 *S. sanguis* cells (50 μl) in CAB and adjusted to a pH of 5.0 with acetate buffer was incubated with 2.75 to 22 μg of adhesin protein at room temperature. After gentle agitation for 30 min, 5× 10^8 *B. loeschei* cells were added to the mixture, which was then shaken for several minutes until the control assay without adhesin added showed strong coaggregation. If the pH of the adhesin solution and the streptococcal cell suspension had been adjusted to 6.8 or higher, the cells quickly aggregated after mixing. The assays were scored visually by assigning values from 0 to +4 or the results were recorded photographically. To establish the specificity of the purified adhesin, controls were performed, using the coaggregation pair *S. sanguis* H1 and *Capnocytophaga ochracea* 25. These assays were performed as described above.

Hemagglutination studies were carried out with sheep erythrocytes (SRBCs) stored in Alsevers solution (19a). SRBCs were washed three times in Hanks buffered saline solution, and packed cells were diluted 1:5 in PBS. SRBCs (1 ml) were treated for 1 h at 37°C with 10 μl of clostridial type X neuraminidase (Sigma Chemical Co., St. Louis, Mo.) containing 10 U of neuraminidase per ml of PBS. SRBCs were washed twice in PBS containing 0.1% bovine serum albumin and 0.02% sodium azide and adjusted to a density of 5 × 10^9 cells per ml in the same buffer. Hemagglutination assays were performed by using microdilution plates; each well contained 80 μl of neuraminidase-treated or untreated SRBCs and 2.5 to 16 μg of purified adhesin (pH 6.8) in a final volume of 140 μl. Controls consisted of untreated cells plus adhesin or treated cells containing buffer only. When necessary, *N*-acetyl-D-galactosamine was added to a final concentration of 6 mM. The microdilution plates were incubated at room temperature with vigorous mixing for 30 min. They were then centrifuged, the size and nature of the resultant pellets were scored visually, and the pellets were photographed. The same protocol was used to test for agglutination of suspensions of *S. sanguis* 34 or *S. sanguis* H1. Suspensions containing 5 × 10^8 cells (in 25 mM sodium acetate, pH 5.6) were incubated with between 5 and 10 μg of

adhesin protein with mixing at ambient temperature. When required, galactosides were added to the agglutination assay at a concentration of 6 mM.

Polyacrylamide gel electrophoresis and isoelectric focusing. Native anionic polyacrylamide gel electrophoresis was carried out on 7% polyacrylamide gel slabs (14 by 13 cm) by using the Tris hydrochloride-glycine buffering system of Davis (5); between 0.5 and 8 μg of protein was added to the sample wells. Denaturing gel electrophoresis was carried out by the method of King and Laemmli (12); similar protein loads were added to the sample wells. Bands on the gels were visualized with Coomassie blue R250 stain (12).

Isoelectric focusing was performed on commercially available pH 3 to 10 ampholine-containing polyacrylamide slabs as specified by the manufacturer (NOVEX Corp., Encinitas, Calif.). Samples containing between 2 and 10 μg of adhesin and a solution containing proteins with known isoelectric points (cytochrome *c* [pI 9.6], chymotrypsin [pI 8.8], whale myoglobin [pI 8.05], equine myoglobin [pI 7.0], human carbonic anhydrase [pI 6.5], bovine carbonic anhydrase [pI 6.0], lactoglobulin B [pI 5.1], and phycocyanin [pI 4.65]) were run concomitantly on the gels. The solution of standard proteins was used to determine the linearity of the pH gradient in the gel, and following staining, the position of the adhesin on the gel was used to estimate its pI.

The native molecular weight of the adhesin was estimated by running anionic-gradient (3 to 20%) polyacrylamide gels to equilibrium (16 to 20 h) by the method of Lambin and Fine (15) and Slater (19b). Oligomeric and monomeric forms of apoferritin (M_r 886,000 and 443,000), urease (M_r 545,000 and 272,000), and bovine serum albumin (M_r 132,000 and 66,000) served as molecular weight standards.

Immunoblot analyses were performed by separating adhesin polypeptide(s) on sodium dodecyl sulfate-gels, transferring the protein components to nitrocellulose filters (1, 20), treating the filters with 1:1,000 dilutions of MAb 3AD6 IgG (3 mg/ml) or 1:2,000 dilutions of polyclonal IgG (5 mg/ml), and visualizing the immune complex with a commercially available alkaline phosphatase goat anti-mouse IgG-conjugated secondary antibody and dye indicator system (Promega Biotec, Madison, Wis.).

N-terminal amino acid sequencing of the adhesin. Lyophilized samples containing between 0.5 and 1 nmol of adhesin were dissolved in 1% trifluoroacetic acid and processed by automated Edman degradations in gas phase sequencer (model 470A; Applied Biosystems, Inc., Foster City, Calif.) in conjunction with the standard NoVac program supplied by the manufacturer. Phenylthiohydantoin derivatives were identified by high-pressure liquid chromatography on an IBM cyano column. The system used with this column consisted of a liquid chromatograph (Series 4; The Perkin-Elmer Corp., Norwalk, Conn.), an LC-85B spectrophotometric detector, and an LCI 100 computing integrator. These procedures were performed under contract with the University of California, San Diego.

Amino acid analysis of the adhesin. Approximately 2 nmol of adhesin was hydrolyzed with constant-boiling 6 N HCl at 110°C for 16 or 24 h. The dried residue was dissolved in Beckman sample buffer diluent and analyzed with a high-pressure liquid chromatography amino acid analyzer (Gold System; Beckman, Inc., Fullerton, Calif.) by using ninhydrin postcolumn derivatization as the detection system. Amino acid standards were obtained from Beckman.

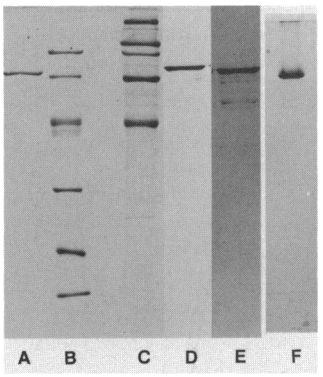

FIG. 1. Demonstration of purity of the streptococcus-specific adhesin by immunoblot analysis, denaturing, and native anionic gel electrophoresis. Lanes A to C show a denaturing sodium dodecyl sulfate-gel. Lane A contains 2 μg of purified adhesin. Lane B contains the following molecular weight standards (top to bottom): phosphorylase *a* (M_r 97,400), bovine serum albumin (M_r 66,200), ovalbumin (M_r 42,700), carbonic anhydrase (M_r 31,000), trypsin inhibitor (M_r 21,400), and lysozyme (M_r 14,400). Lane C contains the following molecular weight standards (top to bottom): myosin (M_r 200,000), β-galactosidase (M_r 116,500), phosphorylase *a*, bovine serum albumin, and ovalbumin. Lane D is an immunoblot developed with MAb 3AD6, containing 0.5 μg of adhesin run in the gel. Lane E is an immunoblot developed with rabbit polyclonal anti-adhesin, containing 0.5 μg of adhesin run in the gel. Lane F, contains 6 μg of adhesin resolved in a native anionic gel (positive electrode at the bottom).

RESULTS

Purification of the *B. loeschei* streptococcus-specific adhesin. The batchwise purification of the adhesin with MAb 5BB1-2 or 3AD6 conjugated to a Sepharose 4B matrix yielded preparations that were greater than 95% pure as determined by scanning laser densitometry of native (Fig. 1, lane F) and denaturing (Fig. 1, lane A) gel electrophoretograms. The minor contaminants which migrated more rapidly than the adhesin monomer, M_r 75,000 (Fig. 1, lanes D and E), reacted with the MAb or polyclonal antiserum specific for the adhesin (Fig. 1, lane C), suggesting that some fraction of the native protein was slightly degraded during the purification procedure. These large fragments of the molecule must have retained the epitope(s) recognized by MAb 5BB1-2 and 3AD6 since they remained associated with the affinity matrix during the purification process and reacted with other MAbs (e.g., 5DB5 and 3BC5 [24]) capable of visualizing the adhesin on immunoblots (data not shown). On nondenaturing anionic polyacrylamide gels (Fig. 1, lane F), the adhesin migrated as a single band remaining near the top of the gel. The relatively slow migration in the electrical field was the first suggestion that the adhesin was a basic protein possessing a net positive charge. The protein aggregated at a pH above neutrality. If the pH of the protein solution was increased to between 7.5 and 8.0, the protein precipitated out of solution and could not be solubilized by making the suspension acidic (pH 3) or by treatment with either 6 M urea or 4% sodium dodecyl sulfate.

Properties of the adhesin. The molecular weight of the native adhesin was estimated by comparing the migration rate of the protein on 3 to 20% gradient anionic polyacrylamide gels with monomeric and polymeric forms of reference proteins. The migration distances were determined at equilibrium; the adhesin migrates like a protein of M_r 450,000

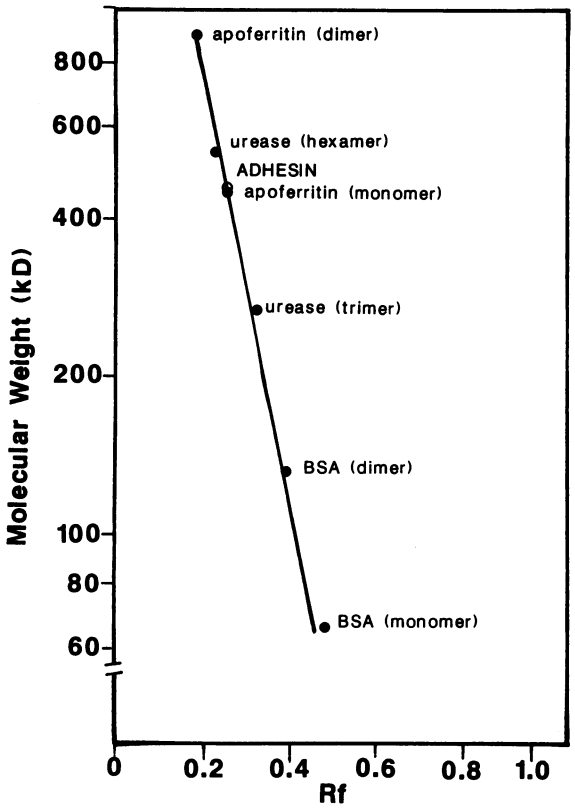

FIG. 2. Estimation of native molecular weight of the streptococcus-specific adhesin by anionic gradient gel electrophoresis. The open circle denotes the relative position of the adhesin in the gel. kD, Kilodaltons.

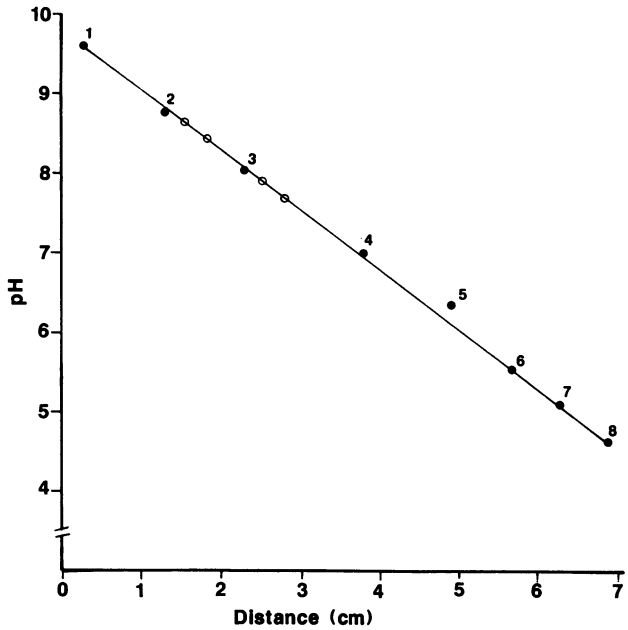

FIG. 3. Distribution of the streptococcus-specific adhesin in a pH gradient. Standards (●) are as follows: 1, cytochrome *c*; 2, α-chymotrypsin; 3, whale myoglobin; 4, equine myoglobin; 5, human carbonic anhydrase; 6, bovine carbonic anhydrase; 7, β-lactoglobin B; 8, phycocyanin. The positions of the adhesin bands (6 μg added, bands of near-equal intensity) are also indicated (○).

(Fig. 2). Thus, in its native form, the adhesin appears to exist as a hexamer. The pI of the adhesin was determined by isoelectric focusing. The affinity-purified adhesin preparations migrated in an apparent polydispersed fashion over a pH range of 7.4 to 8.4 (Fig. 3). The pI of the adhesin in crude preparations was determined after transferring the proteins to nitrocellulose filters; the adhesin was visualized by immunoblot staining (data not presented). These preparations appeared to be less polydispersed and exhibited a pI of between 8.2 and 8.7. Thus, the autoagglutination observed as the pH of the adhesin solution rises above neutrality probably reflects the protein-protein interactions that occur among the adhesin molecules as they approach their pI.

N-terminal amino acid sequencing identified the initial 28 amino acids of the mature protein; this portion of the adhesin was shown to consist of the following amino acid residues: Ala-Tyr-Ser-His-Val-Lys-Asn-Ala-Thr-Gly-Glu-Asp-Ile-Glu-Arg-Ile-Lys-Glu-Glu-Asp-Val-Asp-Asp-Asp-Ile-Glu-Val - Asn. The sequenced segment of the adhesin contains a relatively large number of charged amino acids, including six aspartate residues. In contrast to the overall basic character of the protein, 10 of the N-terminal charged amino acids are acidic. Of the 28 amino acids, only 1 (Tyr) is strongly hydrophobic. The results of an amino acid analysis of the protein adhesin are summarized in Table 1. Glx and Asx account for 22% of the amino acid residues, whereas basic amino acids (Arg, His, and Lys) constitute 13% of the total residues. A significant number of the potentially acidic amino acid residues, i.e., Asx and Glx, probably exist as Asn or Gln, since the pI of the adhesion ranges between 7.4 and 8.4.

Coaggregation inhibition studies. To establish that the purified protein was indeed the streptococcus-specific adhesin, an anti-adhesin polyclonal antiserum was produced in rabbits and its ability to inhibit coaggregation between *B. loeschei* and *S. sanguis* was determined. Since the MAbs originally prepared against the adhesin were potent inhibitors of coaggregation and erythrocyte agglutination by *B. loeschei*, it was reasoned that the polyclonal antiserum would duplicate, at least in part, the action of the MAbs. The purified IgG fraction of the antiserum inhibited the interactions (Fig. 4); however, it was not as effective as the MAbs

TABLE 1. Amino acid analysis of the streptococcus-specific adhesin

Amino acid	No. of residues/ 100 residues
Aspartate[a]	10.8
Glutamate[b]	10.9
Serine	6.8
Glycine	8.0
Histidine	1.2
Arginine	3.9
Threonine	6.0
Alanine	9.0
Proline	4.7
Tyrosine	10
Valine	6.8
Methionine	2.4
Isoleucine	3.0
Leucine	7.2
Phenylalanine	3.6
Lysine	8.0
Cysteine	0.4

[a] Residue may be either Asp or Asn.
[b] Residue may be either Glu or Gln.

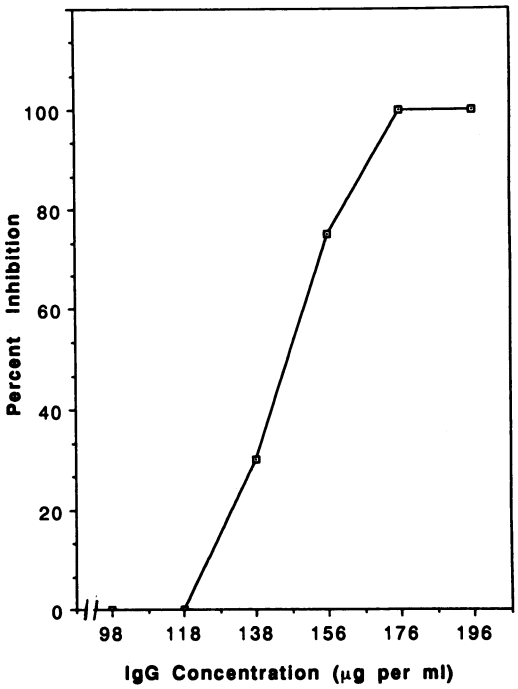

FIG. 4. Inhibition of coaggregation by preincubation of *B. loeschei* cells with polyclonal anti-adhesin prior to addition of *S. sanguis* cells. Preimmune IgG preparations were not inhibitory up to concentrations of 1 mg of protein ml^{-1}.

which inhibited coaggregation in the range of 0.5 to 5 μg of antibody protein (25). IgG prepared from preimmune serum had no effect on the coaggregation reaction at levels of 1 mg of protein ml^{-1}. At similar concentrations (between 98 and 200 μg of IgG protein), the polyclonal antiserum also inhibited hemagglutination of SRBCs (data not shown). In addition, the purified polyclonal IgG reacted with purified preparations of adhesin in a fashion identical to the MAbs on immunoblots (Fig. 1, lane E). The IgG of the polyclonal antiserum functionally resembled the MAbs in that it did not agglutinate *B. loeschei* cells or produce precipitin reactions with crude or purified adhesin preparation in immunodiffusion plates.

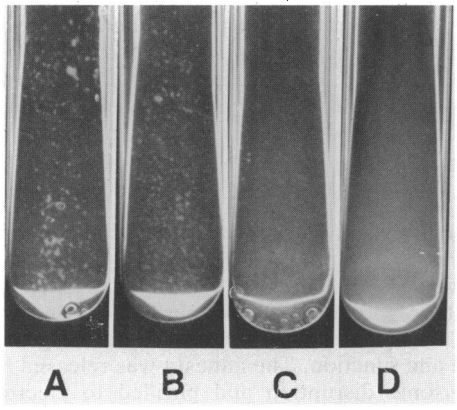

FIG. 5. Inhibition of coaggregation between *B. loeschei* and *S. sanguis* by preincubation of *S. sanguis* cells with streptococcus-specific adhesin. All reaction mixtures contain 5×10^8 *S. sanguis* cells. (A) Control; (B) 5.5 μg of adhesin added; (C) 11 μg of adhesin added; (D) 22 μg of adhesin added.

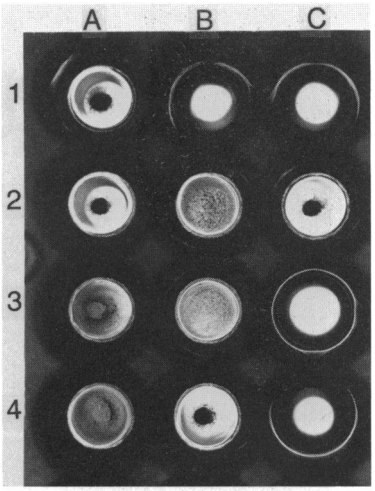

FIG. 6. Hemagglutination of neuraminidase-treated SRBCs by the purified adhesin. Each well contained 5×10^7 cells. (A) Line 1 (control), untreated SRBCs plus 11 μg of adhesin; line 2 (control), treated SRBCs plus PBS buffer; line 3, treated SRBCs plus 2.75 μg of adhesin; line 4, treated SRBCs plus 5.5 μg of adhesin. (B) Line 2, treated SRBCs plus 11 μg of adhesin; line 3, treated SRBCs plus 16.5 μg of adhesin; line 4, treated SRBCs preincubated with 6 mM *N*-acetyl-D-galactosamine plus 11 μg of adhesin. (C) Line 2 (control), treated SRBCs plus 40 μl of acetate buffer.

The purified adhesin itself was capable of inhibiting coaggregation at a low pH (pH 4.6) when preincubated with *S. sanguis* cells prior to the addition of *B. loeschei* cells. Adhesin protein concentrations of 11 to 22 μg (equivalent to 0.2 to 0.4 μmol of native protein) completely inhibited coaggregation between these two microorganisms (Fig. 5C and D). However, constant mixing of the cell suspension containing 11 μg of adhesin protein (Fig. 5C) eventually allowed some coaggregation to occur after 30 min (approximately 25% of the control [Fig. 5A] as estimated visually); at 22 μg, no coaggregation was observed after overnight incubation. Since the concentration of cells used in the assay was known and since blocking occurred at levels between 11 and 22 μg of adhesin, a rough approximation of the number of adhesin molecules required to block each streptococcal cell was determined. An estimated 5.6×10^5 to 11×10^5 molecules per cell resulted in complete abolition of coaggregation. To establish that the effect of the adhesin preparation on coaggregation was specific and not the result of electrostatic interactions between the basic protein and *S. sanguis* cells, a suspension of *S. sanguis* H1 was incubated with 22 μg of adhesin prior to addition of its partner cell, *C. ochracea* 25 (3a). The adhesin had no effect on this reaction (data not shown).

In contrast to the experiment described above, addition of similar levels of purified adhesin preparations (5 to 10 μg; equivalent to a concentration of 0.1 to 0.2 μM of native adhesin) to neuraminidase-treated SRBCs resulted in their rapid agglutination (Fig. 6B, lines 2 and 3). However, it is important to note here that these experiments were carried out at a pH of 6.8 with the adhesin diluted in PBS before being mixed with the SRBCs. No immediate hemagglutination was observed with untreated erythrocytes, but on prolonged incubation (30 to 60 min at room temperature) some formation of particulate material was noted in the reaction mixture (Fig. 6A, line 1). Addition of *N*-acetyl-D-galactosamine at a final concentration of 6 mM to the

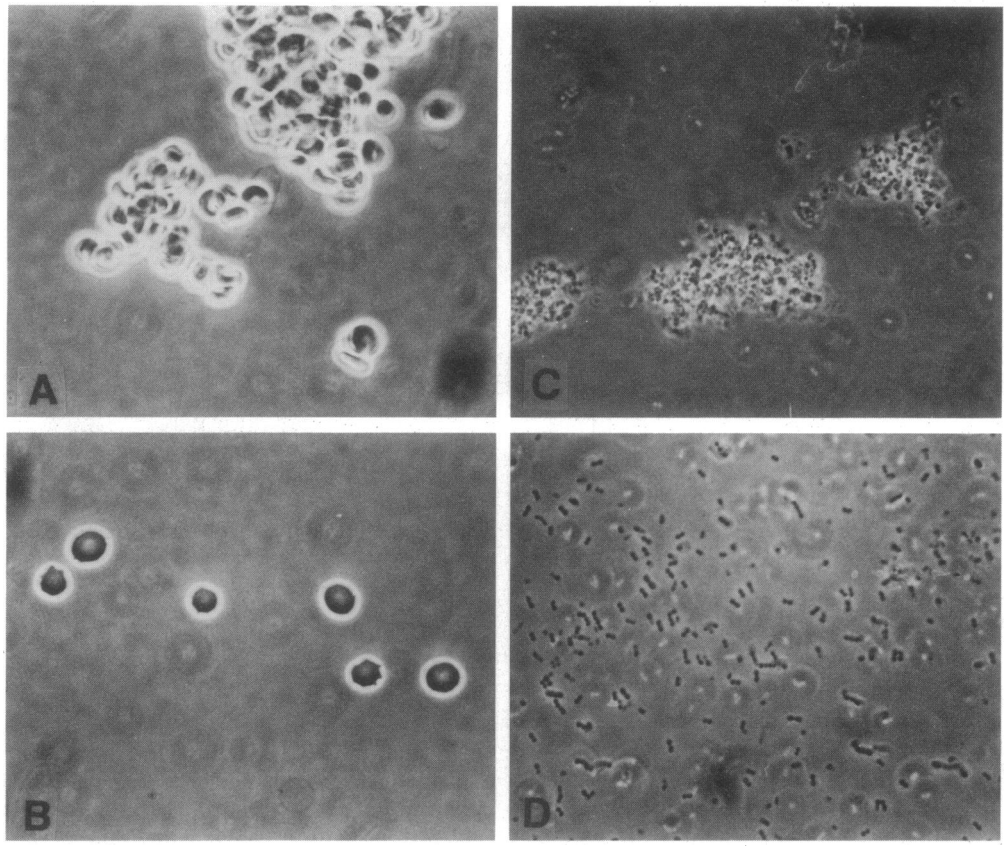

FIG. 7. Adhesin-mediated hemagglutination of SRBCs and aggregation of the *S. sanguis* 34 cells. (A) SRBCs plus 20 μg of adhesin protein; (B) SRBC control (untreated SRBCs plus 20 μg of adhesin); (C) *S. sanguis* cells plus 10 μg of adhesin protein; (D) control (*S. sanguis* cells in buffer only). Magnification, ×480.

SRBCs prior to introducing adhesin prevented hemagglutination (Fig. 6B, line 4). Photomicrographs of reaction mixtures containing adhesin demonstrate conclusively that the adhesin mediates extensive agglutination of the SRBCs (Fig. 7A). If a sample of the neutral adhesin solution (5 μg of protein) was added to *S. sanguis* cells suspended in PBS, the streptococci agglutinated immediately upon mixing (Fig. 7C). The appearance of the suspension was identical to that seen in conventional coaggregation experiments (Fig. 5A and Fig. 8A); photomicrography revealed that, like the SRBCs, the streptococcal cells had undergone extensive aggregation (Fig. 7C). In the controls, adjusted to a pH of 6.8, no aggregation occurred in the absence of adhesin (Fig. 7B and D). As with the SRBCs, addition of galactosides in the same concentration range inhibited or reversed the adhesin-mediated aggregation. Ten micrograms of adhesin failed to aggregate a cell suspension of *S. sanguis* H1, a strain closely related to *S. sanguis* 34, indicating that the interaction was specific for a receptor on the latter (Fig. 8).

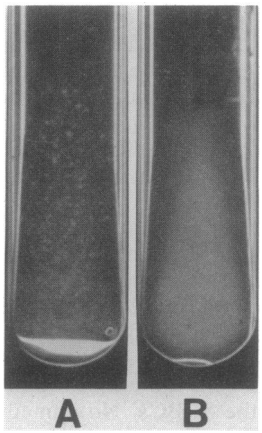

FIG. 8. Specificity of the adhesin-mediated aggregation of streptococcal cells. (A) *S. sanguis* 34 (5×10^8 cells ml^{-1}) plus 10 μg of adhesin protein; (B) *S. sanguis* H1 (5×10^8 cells ml^{-1}) plus 10 μg of adhesin protein. The cells were incubated for 5 min at ambient temperature with mixing.

DISCUSSION

The lectinlike protein on the surface of *B. loeschei* that mediates coaggregation with *S. sanguis* (22) and hemagglutination with a wide variety of erythrocytes (23) is one of the first bacterial adhesins to be purified in any significant quantity and partially characterized with regard to both structure and function. The adhesin was released from cells by ultransonic disruption and purified to electrophoretic homogeneity by affinity chromatography. From its amino acid composition and pI, it appears to be a hydrophilic protein with a comparatively strong positive charge. In its native state, it exists as relatively large hexameric molecule with an estimated M_r of 450,000. The unequivocal identifi-

```
B.l.  A Y C H V K N A T G E D I E R I K E E D V D D D I E V D
Padh  V D N L T F R K L I I P A C T V S N T T V D W Q D V E I
T 1   F A X K T A D G T A I P I G G G X A D V Y V D L A P V V
```

FIG. 9. Comparison of the N-terminal amino acid sequences of *E. coli* type 1 and P adhesins and *B. loeschei* (*B.l.*) adhesin.

cation of its 28 amino-terminal residues suggests that this region of the six monomers making up the native protein is homologous. However, the amino acid sequence of the remainder of the monomer may not be so highly conserved, since isoelectric focusing data indicated that the protein migrates as four distinct bands. The apparent polydispersed nature of the purified preparation may reflect posttranslational modifications of the adhesin monomers or may be a result of acid hydrolysis of the amide groups in glutamine and asparagine during purification. A comparison of N-terminal amino acid sequences of the *E. coli* type 1 (8) and type P (9) adhesins and the *B. loeschei* adhesin showed that each of the *E. coli* proteins matched with the *B. loeschei* protein at two or four nonconsecutive positions (Fig. 9), respectively; the two enteric adhesins showed only six nonconsecutive cross-matches. Thus, there appears to be little or no homology in this portion of the three proteins.

It was essential to establish unequivocally that the protein of M_r 450,000 associated with the *B. loeschei* fimbriae (25; J. London, unpublished data) was the adhesin because the distinction between the adhesin and fimbrillin or pilin subunits in other systems had not been clearly delineated in many earlier studies (for reviews, see references 11 and 13). The following evidence supported the conclusion that the purified protein was the adhesin: (i) the polyclonal antiserum prepared against the adhesin inhibited both coaggregation and hemagglutination; (ii) the purified adhesin itself inhibited coaggregation with streptococcal cells at low pH or agglutinated neuraminidase-treated erythrocytes and streptococci at neutral pH; and (iii) electron micrographs of purified adhesin preparations showed no discernible fimbrial structures (data not presented). The coaggregation inhibition studies established that the adhesin readily binds to partner cells in relatively large numbers. Rough estimates indicated that each cell may bind as many as 4×10^5 adhesin molecules. This number is approximately 3 orders of magnitude greater than the value obtained for the number of adhesin molecules found on individual *B. loeschei* cells (25). It is clear that the adhesin recognizes the same sugars on the procaryote and eucaryote receptors, since *N*-acetyl-D-galactosamine inhibits the blocking of coaggregation and agglutination of streptococcal cells and erythrocytes (23). However, the nature of those receptors is most probably very different. The bacterial receptor may be similar to the *S. sanguis* 34 *N*-acetyl-D-galactosamine-containing cell wall polysaccharide that is recognized by an adhesin on *Actinomyces viscosus* T14V (4), whereas the erythrocyte receptor is probably a glycoprotein or glycolipid (2, 3). *S. sanguis* H1, which failed to interact with the adhesin and, thus, served as an aggregation control, possesses a polysaccharide receptor that is structurally distinct from that described for strain 34 (3a).

The simplest way to account for the ability of the purified adhesin to block coaggregation between *B. loeschei* and *S. sanguis* at pH 4.6 and agglutinate the same streptococcal cells at pH 6.8 is to postulate that the protein starts to aggregate as the pH of the solution approaches the pI of the protein. The tendency for the adhesin to aggregate is probably a function of the protein concentrations, its pI, and the pH of the suspending medium. Thus, although binding activity with streptococcal cells was maintained over the pH range of 4.5 to 7, the physical manifestations of that binding differed markedly. Below pH 5, the adhesin apparently remained in a soluble, nonaggregated state and interacted with streptococcal cells in a manner which blocked the receptor sites of the cell but did not cause agglutination. The ability of adhesin preparations adjusted to pH 6.8 to agglutinate streptococcal cells and neuraminidase-treated SRBCs may reflect the capacity of the adhesin to form soluble aggregates, although none were visible microscopically and centrifugation at 30,000 × *g* for 20 min failed to sediment these putative polyvalent complexes. Blocking experiments with SRBCs were not performed, because the neuraminidase-treated cells became very fragile at pH 5.5 and tended to lyse spontaneously. Once the protein aggregates became visible (pH 7.5 to 8.5), the complex became insoluble, sedimented without centrifugation, and could not be solubilized.

The absence of aggregation at a pH 4.6 does not completely explain the failure of the adhesin to agglutinate streptococcal cells. Since the native adhesin exists as a hexamer composed of monomers of identical molecular weights, the molecule should be polyvalent with respect to its receptor-binding sites. With a molecular mass 2.5 times greater than that of an IgG molecule, some cross-linking between streptococcal cells might have been expected. The absence of formation of an adhesin-mediated lattice work may be a manifestation of how the binding sites are arranged on the monomers of the native molecule, the orientation of the adhesin after its initial binding to the streptococcal cell, and the extent to which the protein structure is flexible. Sufficient adhesin is now available to extensively characterize the physical properties of the adhesin, perform peptide mapping, attempt to identify the protein domain harboring the binding site, and determine the amino acid sequence of the binding site itself. We will also ascertain how the adhesin is associated with the fimbriae.

ACKNOWLEDGMENTS

We gratefully acknowledge Simone Beninati and Ray Fields for their assistance in performing the amino acid analyses of the adhesin. Malcom Moos graciously confirmed the N-terminal amino acid sequence, and Paul E. Kolenbrander, John Thompson, and John Folk provided helpful criticism during the preparation of this manuscript.

LITERATURE CITED

1. **Batteiger, B., W. J. Newhall, and R. B. Jones.** 1982. The use of Tween 20 as a blocking agent in the immunological detection of proteins transferred to nitocellulose membranes. J. Immunol. Methods **55**:297–307.
2. **Brennan, M. J., J. O. Cisar, A. E. Vatter, and A. L. Sandberg.** 1984. Lectin-dependent attachment of *Actinomyces naeslundii* to receptors on epithelial cells. Infect. Immun. **46**:459–464.
3. **Brennan, M. J., R. A. Joralmon, J. O. Cisar, and A. L. Sandberg.** 1987. Binding of *Actinomyces naeslundii* to glycosphingolipids. Infect. Immun. **55**:487–489.

3a. **Cassels, F. J., and J. London.** 1989. Isolation of a coaggregation-inhibiting cell wall polysaccharide from *Streptococcus sanguis* H1. J. Bacteriol. **171**:4019–4025.
4. **Cisar, J. O., M. J. Brennan, and A. L. Sandberg.** 1985. Lectin-specific interaction of *Actinomyces* fimbriae with oral streptococci, p. 159–163. *In* S. E. Mergenhagen and B. Rosan (ed.), Molecular basis of oral microbial adhesion. American Society for Microbiology, Washington, D.C.
5. **Davis, B. J.** 1964. Disc electrophoresis. II. Method and application to human serum. Ann. N.Y. Acad. Sci. **12**:404–427.
6. **Duguid, J. P., and R. R. Gillies.** 1957. Fimbriae and adhesive properties in dysentery bacilli. J. Pathol. Bacteriol. **74**:397–411.
7. **Duguid, J. P., I. W. Smith, G. Dempster, and P. N. Edmunds.** 1955. Non-flagellar filamentous appendages ("fimbriae") and hemagglutinating activity in *Bacillus coli*. J. Pathol. Bacteriol. **70**:335–348.
8. **Hanson, M. S., J. Hempel, and C. C. Brinton, Jr.** 1988. Purification of the *Escherichia coli* type 1 pilin and minor pilus proteins and partial characterization of the adhesin protein. J. Bacteriol. **170**:3350–3358.
9. **Hoschutzky, H., F. Lottspeich, and K. Jann.** 1989. Isolation and characterization of the α-galactosyl-1,4-β-galactosyl adhesin (P adhesin) from fimbriated *Escherichia coli*. Infect. Immun. **57**: 76–81.
10. **Ingraham, J. L., O. Maaloe, and F. C. Neidhart.** 1983. Growth of the bacterial cell, p. 3. Sinauer Associates, Boston.
11. **Jones, G. W., and R. E. Isaacson.** 1983. Proteinaceous bacterial adhesins and their receptors. Crit. Rev. Microbiol. **10**:229–260.
12. **King, J., and U. K. Laemmli.** 1971. Polypeptides of tail fibers of bacteriophage T4. J. Mol. Biol. **62**:465–477.
13. **Klemm, P.** 1985. Fimbrial adhesins of *Escherichia coli*. Rev. Infect. Dis. **7**:321–340.
14. **Kolenbrander, P. E.** 1988. Intergeneric coaggregation among human oral bacteria and ecology of dental plaque. Annu. Rev. Microbiol. **42**:627–656.
15. **Lambin, P., and J. M. Fine.** 1979. Molecular weight estimations of proteins by electrophoresis in linear polyacrylamide gradient gels in the absence of denaturing agents. Anal. Biochem. **98**:160–168.
16. **Lund, B., F. Lindberg, B.-I. Marklund, and S. Normark.** 1987. The PapG protein is the α-D-galactopyranosyl-(1-4)-β-D-galactopyranoside-binding adhesin of uropathogenic *Escherichia coli*. Proc. Natl. Acad. Sci. USA **84**:5898–5902.
17. **Lund, B., F. Lindberg, and S. Normark.** 1988. Structure and antigenic properties of the tip-located P pilus proteins of uropathogenic *Escherichia coli*. J. Bacteriol. **170**:1887–1894.
18. **McIntire, F. C., A. E. Vatter, J. Baros, and J. Arnold.** 1978. Mechanism of coaggregation between *Actinomyces viscosus* T14V and *Streptococcus sanguis* 34. Infect. Immun. **21**:978–988.
19. **Mooi, F. R., M. van Buuren, G. Koopman, B. Roosendaal, and F. K. de Graaf.** 1984. K88ab gene of *Escherichia coli* encodes a fimbrialike protein distinct from the K88ab fimbrial adhesin. J. Bacteriol. **159**:482–487.
19a. **Rapp, H. J., and T. Borsos.** 1970. Molecular basis of complement action, p. 78. Appleton-Century-Crofts, Meredith Corp., Englewood Cliffs, N.J.
19b. **Slater, G. G.** 1969. Stable pattern formation and determination of molecular size by pore limit electrophoresis. Anal. Chem. **41**:1039–1041.
20. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
21. **Uhlin, E. B., M. Norgren, M. Baga, and S. Normark.** 1985. Adhesion to human cells by *Escherichia coli* lacking the major subunit of a digalactose-specific pilus-adhesin. Proc. Natl. Acad. Sci. USA **82**:1800–1804.
22. **Weiss, E. I., P. E. Kolenbrander, J. London, A. R. Hand, and R. N. Andersen.** 1987. Fimbria-associated proteins of *Bacteroides loeschei* PK1295 mediate intergeneric coaggregations. J. Bacteriol. **169**:4215–4222.
23. **Weiss, E. I., J. London, P. E. Kolenbrander, and R. N. Andersen.** 1989. Fimbria-associated adhesin of *Bacteroides loeschei* that recognizes receptors on procaryotic and eucaryotic cells. Infect. Immun. **57**:2912–2913.
24. **Weiss, E. I., J. London, P. E. Kolenbrander, R. N. Andersen, C. Fischler, and R. P. Siraganian.** 1988. Characterization of monoclonal antibodies to fimbria-associated adhesins of *Bacteroides loeschei* PK1295. Infect. Immun. **56**:219–224.
25. **Weiss, E. I., J. London, P. E. Kolenbrander, A. R. Hand, and R. P. Siraganian.** 1988. Localization and enumeration of fimbria-associated adhesins of *Bacteroides loeschei*. J. Bacteriol. **170**:1123–1128.
26. **Williams, C. A., and M. W. Chase.** 1967. Purification of antibody. Methods Immunol. Immunochem. **3**:321–326.