Identification and Preliminary Characterization of a Streptococcus sanguis Fibrillar Glycoprotein

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Cell surface fibrils could be released from Streptococcus sanguis 12 but not from strains 12na or N by freeze-thawing followed by brief homogenization. Fibrils were isolated from the homogenate by ultracentrifugation or ammonium sulfate precipitation. Electron microscopy demonstrated the presence of dense masses of aggregated fibrils in these preparations. Under nondenaturing conditions, no proteins were seen in polyacrylamide gel electrophoresis (PAGE). Sodium dodecyl sulfate (SDS)-PAGE analysis revealed a single band stained with Coomassie blue and periodic acid Schiff stain with a molecular weight in excess of 300,000. The protein has been given the name long-fibril protein (LFP). The molecule was susceptible to digestion with subtilisin, pronase, papain, and trypsin, but was unaffected by chymotrypsin or muramidases. Attempts to dissociate the protein into smaller subunits with urea, guanidine, sodium thiocyanate, and HCl were unsuccessful. Gel filtration on a column of Sephacryl S-400 in 2% SDS resulted in elution of the protein at the void volume. Antibody raised against the LFP excised from an SDS-PAGE gel reacted with long fibrils on the surface of strain 12 and with isolated fibrils by an immunogold labeling technique. Monoclonal antibody reactive with LFP in SDS-PAGE also reacted with fibrils present on the cell. Antisera raised against the fibrils inhibited adherence to saliva-coated hydroxyapatite.

Cell surface structures variously called fibrils, fimbriae, and pili have been identified on numerous gram-negative microorganisms (21, 40) but have been found on only a limited range of gram-positive organisms, including Corynebacterium renale (24) and Actinomyces spp. (3, 26). Electron microscopy has also revealed fibrillar structures (earlier termed fuzzy coats) on a number of species of streptococci, including Streptococcus salivarius (16), group A streptococci (1, 8, 43), Streptococcus faecalis (18), and Streptococcus sanguis (4, 12, 13, 17, 19, 27, 36). Handley et al. (16) define fibrils as structures extending from the cell surface which have a defined length but do not have a consistent width, presumably because they are clumped. Fimbriae have a defined width and length both. Short and long fibrils have been identified on the surface of S. sanguis 12 (34).

Fimbrial or fibrillar structures on the surfaces of many microorganisms are known to function in bacterial adhesion (21). Attempts have therefore been made to correlate the surface structures of streptococci with various adhesive properties. These include coaggregation with other bacteria (13, 17, 36), salivary aggregation (13, 20), hemagglutination (20), and adherence to saliva-coated hydroxyapatite (S-HA) (12, 13, 15). Fachon-Kalweit et al. (11) recently demonstrated that an antifimbrial antibody would inhibit adherence of S. sanguis FW-213 to S-HA. Little has been done to characterize the surface fibrillar molecules of S. sanguis. Nagata et al. (38) isolated a galactose-binding lectin with hemagglutinating activity from S. sanguis 10557 which was probably associated with fibrils. This had a molecular weight (MW) of 20,000. Although considerable effort has gone into identifying other adhesins on S. sanguis (30, 37), a possible fibrillar location for such molecules remains to be determined.

Weerkamp et al. (47) have used gold-labeling techniques to show that high-molecular-weight (HMW) proteinaceous adhesins of S. salivarius are associated with or synonymous to specific classes of fimbriae. HMW proteins have also been implicated in the adherence of S. sanguis (34) and S. mutans (31).

This paper describes the identification and preliminary characterization of an HMW fibrillar glycoprotein from the surface of S. sanguis 12.

MATERIALS AND METHODS

Bacteria. S. sanguis 12 and N adhere to S-HA, aggregate in saliva, and bind to hexadecane. S. sanguis 12na and 12L are variants derived from strain 12. Strain 12na does not aggregate in saliva, but adheres to S-HA and hexadecane. S. sanguis 12L shows reduced binding to hexadecane; does not aggregate in saliva, and does not adhere to S-HA. These strains have been described in detail previously (32, 34). Cells were grown in trypticase soy broth (BBL Microbiology Systems) supplemented with yeast extract (3 g liter−1) in a 100-liter fermentor. Medium constituents were mixed and sterilized in situ. Following sterilization, the medium was rapidly cooled to 37°C and inoculated with 2.0 liters of an overnight culture of S. sanguis. The culture was incubated at 37°C without stirring until the cells had reached the stationary growth phase. Cells were harvested in a continuous-flow centrifuge (Sharples, Pennawalt). Cell yields were approximately 2.0 g (wet weight) per liter. In some cases cells were grown in the same medium in 20-liter glass bottles. All strains showed similar growth rates under these culture conditions.

Hydrophobicity and salivary aggregation titers of the bacteria were determined as described previously (30, 33). Isolation of fibrils. Fibrils were removed from the cells by a shearing technique. Cells from a 20-liter culture were harvested by centrifugation, washed once in buffer (20 mM Tris, 1 mM MgCl2, 0.02% NaN3, pH 6.8), and frozen as a pellet at −20°C. The pellet was thawed, washed once in Tris-MgCl2 (pH 6.8) buffer, and then suspended in 200 ml of
the same buffer. The cell suspension was homogenized in a Waring blender twice for 1 min each, after which the cells were removed by centrifugation and the supernatant, containing the fibrils, was retained. In initial experiments the fibrils were recovered by centrifugation. The crude extract from homogenization was lyophilized and redissolved in 20 ml of Tris-MgCl₂ buffer and then centrifuged at 80,000 x g for 2 h. The precipitate was suspended in 20 ml of Tris-MgCl₂ buffer and centrifuged at 12,000 x g for 30 min to remove any cells or cell fragments. The supernatant was then centrifuged again at 80,000 x g for 2 h. The fibrillar preparation was obtained as a yellowish, gel-like, translucent pellet which was suspended in 2.5 ml of Tris-MgCl₂ buffer. In later experiments fibrils were isolated from the crude homogenate by ammonium sulfate precipitation. Ammonium sulfate was added to an initial concentration of 15% and incubated at 4°C overnight. Precipitated material was removed by centrifugation at 15,000 x g for 40 min. The ammonium sulfate concentration was then raised to 30%, and the mixture was incubated at 4°C overnight. At this concentration the fibrils were precipitated. The precipitate was recovered by centrifugation, dialyzed, and suspended in Tris-MgCl₂ buffer as before.

**Gel filtration.** The fibrillar preparation was dialyzed against distilled water, freeze-dried, and suspended in 0.05 M HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer, pH 8.0, containing 2% sodium dodecyl sulfate (SDS). The sample was then applied to a column (30 by 1.6 cm) of Sephacryl S-400. The column was eluted with the same buffer. Fractions (1 ml) were collected and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE).

**Electrophoretic techniques.** PAGE and SDS-PAGE were run with 7% polyacrylamide gels (25) unless stated otherwise in the text. Samples for SDS-PAGE were boiled in 2% SDS and 5% β-mercaptoethanol (SDS-BME) for 5 min unless stated otherwise. MW standards were myosin (200,000), β-galactosidase (116,250), phosphorylase B (92,500), bovine serum albumin (66,200), and ovalbumin (45,000). Gels were stained for carbohydrate with periodic acid-Schiff reagent (49) and for protein with Coomassie blue or silver nitrate (39). Alternatively, proteins were transferred electrophoretically to nitrocellulose paper for Western blotting (2) in 25 mM Tris–192 mM glycine–20% methanol buffer (pH 8.3). A voltage of 25 V was applied for 18 h in a Bio-Rad Trans-Blot cell. The voltage was then increased to 60 V for 2 h. Proteins which reacted with specific antisera were visualized on the nitrocellulose by the procedure described in the Bio-Rad Immun-Blot (GAR-HRP or GAM-HRP) assay kit, except that bovine serum albumin (BSA) was substituted for gelatin in the procedure.

ELISA. Fibrillar preparations in carbonate coating buffer (0.05 M, pH 9.6) were dried onto microtiter plates (Immulon 2; Dynatech Laboratories Inc., Alexandria, Va.) by overnight incubation at 37°C.

The enzyme-linked immunosorbent assay (ELISA) was performed as described previously (9, 44). Coated plates were washed with phosphate-buffered saline containing 0.01% Tween 20 (PBS-Tween). Free sites on the plates were blocked by reaction with 5% BSA for 30 min, followed by a further wash with PBS-Tween. Antibody at an appropriate dilution in PBS with 1% BSA was added to the wells (0.1 ml per well) and incubated for 2 h at room temperature. This was followed by washing with PBS-Tween and then incubation with the alkaline phosphatase-labeled rabbit immunoglobulin G (IgG) or goat anti-mouse immunoglobulin (both from Helix Biotech Ltd.) at a dilution of 1:6,000 in PBS–1% BSA. A final wash with PBS-Tween was followed by the addition of 0.1 ml of p-nitrophenyl phosphate substrate (Sigma Chemical Co., St. Louis, Mo.) to each well. Color development was monitored after 1 h of incubation at 37°C by measurement of the absorbance at 405 nm on a Titertek Multiscan (Flow Laboratories).

**Antisera.** (i) **Anti-LF antibody.** To obtain anti-long-fibril protein (LFP) antibody, rabbits were immunized with purified protein from an SDS-PAGE gel. Gels were stained with Coomassie blue, and the required band was excised from the gel. This strip of gel was completely destained in 25% isopropanol–10% acetic acid and then equilibrated overnight in PBS. The gel was homogenized in PBS and injected intramuscularly on days 1, 7, and 28. Booster injections were given at approximately monthly intervals thereafter.

(ii) **Anti-fibrill antibody.** Rabbits were immunized by intramuscular injection with a fibrillar preparation in complete Freund adjuvant. This was followed on days 7 and 28 with intramuscular injections of fibrillar preparation in incomplete Freund adjuvant. Booster injections were given at monthly intervals thereafter.

For all subsequent immunological procedures, antisera was passed through a column of protein A-Sepharose CL4B (Sigma Chemical Co.) to obtain purified IgG. The sample was exhaustively washed on the column with 0.1 M borate–0.5 M NaCl buffer, pH 8.4. IgG was then eluted with 0.1 M glycine–0.5 M NaCl buffer, pH 2.5, followed by dialysis against an appropriate buffer as required for subsequent use.

**Monoclonal antibody production.** Monoclonal antibody (Mab) was produced as described by Kohler and Milstein (23) and Geffer et al. (14). Mice were given an initial immunization by intraperitoneal injection of live cells of S. sanguis 12 (10⁵ cells per mouse) mixed 1:1 with Freund complete adjuvant. After 1 month, the mice were injected with the LFP eluted from an SDS-PAGE gel (see antisera section above). After three days, spleen cells were removed and fused with myeloma line Sp2/0 in polyethylene glycol 1550. The resulting hybridomas were screened in an ELISA for antibody reactive against a fibrillar preparation from S. sanguis 12. The cells were cloned by limiting dilution and retested for antibody activity by ELISA. Culture supernatants from hybridomas which continued to show a positive reaction were tested by Western blot analysis for reaction with the LFP band. One Mab, 32.33, which reacted strongly with the LFP band in a Western blot was used to obtain the results described in this paper.

**Electron microscopy.** (i) **Negative staining.** Whole cells or fibrillar preparations were negatively stained with 5% uranyl acetate or with 2% (wt/vol) phosphotungstic acid adjusted to pH 7.2 with KOH. Observations were made with a Philips EM300 electron microscope.

(ii) **Immunogold technique.** Gold beads (18 nm diameter) were prepared as described by Robinson et al. (41). These were coated directly with IgG as described by Mouton and Lamonde (35). The coated beads were suspended in 20% of the original volume of 20 mM Tris–500 mM NaCl, pH 7.5. Samples (bacteria or fibrillar preparations) were labeled with the coated beads on electron microscope grids essentially as described by Robinson et al. (41). Specimens were then negatively stained as described above. Beads coated with nonimmune IgG were included as a control.

**Bacterial adherence to S-HA.** Adherence was determined by the method of Morris and McBride (33). S-HA was incubated overnight at pH 5.0 and 37°C before addition of bacteria. When indicated, bacteria were preincu-
bated with F(ab')2 fragments (final concentration, 1 mg of protein per ml) for 1 h at room temperature prior to their addition to S-HA. Unbound F(ab')2 was removed by centrifugation.

**Enzyme digests.** Fibrillar preparations were digested at 37°C for 1 h or overnight in buffer (20 mM Tris, 1 mM MgCl2, 0.02% NaN3, pH 6.8) with trypsin, chymotrypsin, papain, subtilisin, lysozyme, or pronase at a concentration of 1 mg/ml or with mutanolysin at a concentration of 175 U/ml. Mutanolysin, lysozyme, trypsin, papain, and subtilisin were from Sigma. Chymotrypsin was obtained from Worthington Diagnostics, Freehold, N.J., and pronase was obtained from Calbiochem-Behring, La Jolla, Calif.

**Dissociation of fibrils.** Attempts were made to dissociate the fibrils into smaller subunits by treatment with a variety of chemical agents. Fibrillar preparations were dialyzed, freeze-dried, and then treated with 8 M urea, pH 7, 8.6 M guanidine hydrochloride, pH 7, 54 mM HCl (pH 1.8), or 1 M sodium thiocyanate, pH 7; 0.1% EDTA was included with each treatment done at neutral pH to prevent possible reaggregation of fibrillar subunits (22).

All treatments were carried out at 37°C overnight or at 100°C for 15 min. Reagents were removed by dialysis against water containing 0.1% EDTA before analysis by SDS-PAGE.

**Analytical procedures.** Protein concentrations were measured with the Bio-Rad protein assay (Bio-Rad Laboratories) with BSA or bovine gamma globulin as the standard. Total carbohydrate was measured by the phenol-sulfuric acid method (6) with glucose as the standard. Amino acid analyses were performed by high-pressure liquid chromatography with a Waters sulfonated polystyrene cation exchange resin. Samples were hydrolyzed with 6 N HCl in vacuo for 24 h at 110°C. After removal of the HCl, the sample was suspended in 0.2 M sodium citrate, pH 2.2, for injection onto the column. Amino acids were eluted with a linear gradient of 0.2 M sodium citrate, pH 3.0, to 0.2 M sodium borate, pH 9.8. Eluted amino acids were detected by post-column reaction with α-phthalaldehyde and monitored by fluorescence. Amino acids containing secondary amines were detected by hypochlorite oxidation prior to α-phthalaldehyde derivatization.

**F(ab')2 preparation.** Protein A-Sepharose CL4B-purified IgG was digested with pepsin at a concentration equal to 2% of total IgG by weight. The digestion was carried out in 0.1 M sodium acetate buffer (pH 4.5) at 37°C for 18 h and stopped by adjusting the pH to 8.0 with 1 M Tris. Pepsin was obtained from Sigma. The digest was assayed for F(ab')2 fragments by SDS-PAGE.

**RESULTS**

**Fibrillar preparations.** The presence of fibrils of *S. sanguis* 12 prepared by ultracentrifugation or ammonium sulfate precipitation after freeze-thawing and homogenization was confirmed by electron microscopy of negatively stained preparations. Fibrillar preparations appeared turbid, and electron microscopy revealed the presence of large aggregates of fibrils and relatively few individual fibrils (Fig. 1). Electron microscopy of preparations obtained by the same treatment of *S. sanguis* 12na and *S. sanguis* N did not show any fibrillar structures.

The preliminary freezing of cells appeared to be essential for the subsequent release of fibrils, because no fibrils were obtained when this step was omitted. Bacteria examined in the electron microscope after freezing and homogenization still possessed considerable fibrillar material on their cell surfaces. These cells retained their hydrophobicity but showed a somewhat reduced salivary aggregation titer. For harvested cells, 91% adhered to hexadecane and the salivary aggregation titer was 256; for frozen and homogenized cells, adherence was 90% and the titer was 64.

In early studies, glucose was sterilized separately and added to the sterilized growth medium after cooling. Cells harvested from this medium had a reduced ability to bind to S-HA and yielded only small amounts of fimbriae. In addition, the fimbrial preparations contained large quantities of contaminating material which was not seen in preparations obtained from cells grown in medium prepared as described in Materials and Methods.

**Composition of fibrillar preparations.** Fibrillar preparations from *S. sanguis* 12 were found to contain approximately equal quantities of protein and carbohydrate. The recovery of both protein and carbohydrate was 3 μg/g (wet weight) of cells. ELISA with antiserum raised against lipoteichoic acid (LTA) from *Lactobacillus casei* (anti-LTA serum was a generous gift from K. Knox, Institute of Dental Research, United Dental Hospital of Sydney, Surry Hills, New South Wales, Australia 2010) indicated that fibrillar preparations did not contain LTA.

**Characterization of the fibril.** There were no silver-staining bands when the fibrillar preparation was analyzed by 7% polyacrylamide gel electrophoresis (PAGE) under nondenaturing conditions. However, SDS-PAGE analysis of fibrillar preparations of *S. sanguis* 12 showed a single strong band with an MW greater than 300,000, with very little contaminating material (Fig. 2). Similar results were obtained with both the ultracentrifugation and ammonium sulfate purification procedures. This is referred to subsequently as the LFP band. Fibrillar preparations were routinely boiled in 2% SDS–5% BME prior to PAGE analysis. However, boiling was not required to dissociate the protein, as fibrils mixed with 2% SDS at room temperature were dissociated into a component which migrated into the gel. When the SDS-treated fibrillar preparation was fractionated on Sephacryl S-400, the LFP was found to elute in the void.

**FIG. 1.** Electron micrograph of fibrillar preparation from *S. sanguis* 12 obtained by ultracentrifugation. Negatively stained with phosphotungstic acid. Bar, 0.5 μm.
volume, indicating an MW in excess of 300,000. The LFP band was not found in preparations obtained from strains 12na and N (Fig. 2). A band of MW 145,000 was seen in the preparation from 12na. A comparable band of varying intensity was sometimes seen in fibrillar preparations from \textit{S. sanguis} 12 obtained by the ultracentrifugation method. When ammonium sulfate precipitation was used, this band was found in the material which precipitated at 15% saturation. A band of MW 160,000 also precipitated at 15% saturation, as well as bands of lower MW (34).

The LFP band stained positively with both Coomassie blue and periodate-Schiff, indicating that it was a glycoprotein. Both staining activities were lost after incubation of the fibrillar preparation with subtilisin (1 mg/ml) for 5 min. Information on the chemical nature of the fibrils was sought by digesting purified fibrillar preparations with a number of hydrolytic enzymes (Fig. 3). The fibrils were incubated with the enzyme, and at appropriate intervals samples were removed from the digestion mixture and immediately boiled in SDS-BME solubilization mixture. The SDS-treated samples were then analyzed by SDS-PAGE. Subtilisin, pronase, and papain completely hydrolyzed the silver nitrate and Schiff-periodate-staining bands which we have described as the LFP. Exposure of the fibrils to 100 \( \mu \)g of subtilisin for 5 min was sufficient to cause complete loss of the protein. Incubation with lower amounts of the enzyme reduced the rate at which the LFP disappeared, but at no time were any degradation products visible in silver nitrate-stained gels. This was the case even when samples were applied to 5 to 20% gradient gels. The fibrils were moderately sensitive to trypsin; incubation with 1 mg of the enzyme led to the formation of a broad lower-MW silver-staining region. Chymotrypsin did not hydrolyze the glycoprotein. The fibrils were insensitive to mutanolysin and lysozyme, suggesting that peptidoglycan is not associated with the glycoprotein.

(FIG. 3). Amino acid analysis of the fibrils is shown in Table 1.

\textbf{Immunological analysis.} The fibrillar preparation was subjected to SDS-PAGE and then examined for its ability to react with a variety of antiserum (Fig. 4). Antisera raised against either formalinized whole cells (34) or the fibrillar preparation reacted with the LFP. The reaction of LFP with anti-12 serum could be eliminated by absorbing the serum with whole cells of \textit{S. sanguis}. This suggests that the LFP is located on the bacterial cell surface. Antisera raised against

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Content in whole fibril (mol%)</th>
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<tbody>
<tr>
<td>Aspartic acid</td>
<td>9.3</td>
</tr>
<tr>
<td>Threonine</td>
<td>6.4</td>
</tr>
<tr>
<td>Serine</td>
<td>14.5</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>15.7</td>
</tr>
<tr>
<td>Proline</td>
<td>3.6</td>
</tr>
<tr>
<td>Glycine</td>
<td>15.0</td>
</tr>
<tr>
<td>Alanine</td>
<td>9.9</td>
</tr>
<tr>
<td>Cysteine</td>
<td>0</td>
</tr>
<tr>
<td>Valine</td>
<td>5.1</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.3</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>3.6</td>
</tr>
<tr>
<td>Leucine</td>
<td>4.8</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>2.2</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>2.3</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.7</td>
</tr>
<tr>
<td>Lysine</td>
<td>3.3</td>
</tr>
<tr>
<td>Arginine</td>
<td>2.0</td>
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</table>
LFP which had been eluted from a gel also reacted with the protein (Fig. 4, lane 3). No reaction was seen with any protein in culture supernatants from strain 12 or from the nonaggregating (12na) or nonadhering (12L) strains. Mab 32.33, derived from mice immunized with whole cells of S. sanguis, reacted with the LFP (Fig. 4). This Mab also reacted with the fibrillar preparation, as determined by ELISA.

**Immunogold electron microscopy.** S. sanguis 12 possesses two lengths of surface fibrils, as shown in Fig. 5. The shorter layer, which often appeared as an indistinct capsular material, was approximately 70 nm long. The longer fibrils, which were more prevalent at the poles, had a length of at least 200 nm. IgG raised against the LFP band and coated on gold beads reacted strongly with the long surface fibrils. Figure 6 shows the beads clearly aligned along the long fibrils, while they apparently did not adhere to or were unable to penetrate the short fibrillary layer, seen only indistinctly in Fig. 6.

The ability of anti-LFP-coated gold beads to bind to S. sanguis 12na was also studied. Although no fibrils could be isolated from this strain, which has lost the ability to aggregate in saliva and has altered adherence properties (34), some adherence of gold beads occurred to a surface layer around 12na cells (Fig. 7). The position of the beads suggested that they were located at the external ends of the short fibrillar layer seen on strain 12. The beads often adhered

![FIG. 4. Western blot of fibrillar preparation of S. sanguis 12 with different antibodies. Lanes: 1. anti-S. sanguis 12; 2. antifibril; 3. anti-LFP; 4. Mab 32.33. The LFP did not react with nonimmune IgG or IgM. Arrow indicates reaction with LFP.](http://jb.asm.org/)

![FIG. 5. Electron micrograph of S. sanguis 12 negatively stained with uranyl acetate, showing two lengths of fibrils on the cell surface. Bar, 0.5 μm.](http://jb.asm.org/)

![FIG. 6. Cells of S. sanguis 12 reacted with gold beads coated with antiserum raised against the LFP band excised from an SDS-PAGE gel. Negatively stained with uranyl acetate. Bar, 0.5 μm.](http://jb.asm.org/)

![FIG. 7. Cells of S. sanguis 12na reacted with gold beads as in Fig. 6. Bar, 0.5 μm.](http://jb.asm.org/)
more densely at the poles of the cell, as seen in Fig. 7; this may represent adherence to some residual or defective form of the long fibrils.

Gold beads coated with antibody to the LFP protein adhered in large numbers to aggregated masses of fibrils in the fibrillar preparations, suggesting that these fibrils were the same as the long fibrils on the cell surface of strain 12. Gold beads coated with nonimmune IgG did not adhere either to whole cells of strain 12 or to isolated fibrils.

Gold beads coated with the LFP-reacting Mab 32.33 formed a halo around the cells (Fig. 8), suggesting that Mab 32.33 recognized an epitope in the terminal subunit of the fibril. The position of the gold bead suggests that it was at a point consistent with the end of a long fibril.

Dissociation of fibrils. It was considered possible that the LFP might be made up of subunits, as found for the fimbrial proteins of a number of gram-negative microorganisms (21, 22). Consequently, various dissociating agents were tested for their ability to break down the LFP band into smaller units. Urea (8 M), guanidine hydrochloride (8.6 M), and sodium thiocyanate (1 M) were found to be completely ineffective. HCl treatment caused partial disappearance of the band when the sample was boiled in HCl at pH 1.8 for 5 to 15 min and total disappearance after overnight incubation at 37°C. However, no bands of lower MW were formed which were visible in either a 7 or 12% silver-stained gel. Boiling the fibrillar preparation in SDS-BME for 1 h instead of 5 min prior to SDS-PAGE analysis resulted in some degradation of the protein. A long silver-staining smear was seen on the gel, but discrete subunits could not be detected. The smear reacted with anti-LFP serum.

S-HA binding. The possibility that the fibrils were involved in binding to S-HA was assessed in an indirect antibody-blocking assay. As seen in Table 2, F(ab′)2 fragments prepared from antifibrillar antibody inhibited binding by 85%. F(ab′)2 fragments prepared from antisera raised against the LFP which had been excised from an SDS-PAGE gel did not inhibit binding. This is probably because antibody was raised against a denatured protein which did not express the appropriate epitopes. The F(ab′)2 fragments prepared from nonimmune IgG had no effect. Mab 32.33 is of the IgM class and was found to cause extensive aggregation of the cells. The formation of aggregates precluded testing the antibody for its ability to block adherence.

**DISCUSSION**

The technique of freezing followed by homogenization has provided a means of releasing fibrils from the surface of *S. sanguis* 12. The technique used resembles that of Nagata et al. (38), who repeatedly froze and thawed their cells. It seems likely that our technique may release only a particular class of fibrils, which are not universally distributed among strains of *S. sanguis*, since no fibrils were obtained from *S. sanguis* N that could be seen in the electron microscope to possess surface fibrils. It was less surprising that fibrils were not obtained from strain 12na, since it is known that this strain has lost a number of cell surface molecules (34). An alternative explanation for the failure to release fibrils from strain N might be that the fibrils are linked more tightly to the cell wall and cannot be removed as easily from this strain.

The observation that the fibrillar preparation contained only a single protein of MW greater than 300,000 when analyzed by SDS-PAGE represents the first positive identification of a fibrillar molecule from *S. sanguis*. Its proteinaceous nature was confirmed by its susceptibility to some proteolytic enzymes. Immunogold electron microscopy with antibody raised against this protein confirmed that it was indeed associated both with fibrils present on whole cells of 12 and with isolated fibril preparations. This was substantiated by the observation that Mab reacted with both the LFP and the fibrils.

The purified LFP was not degraded by mutanolysin when incubated with the enzyme for extended periods. The fibrillar protein was released from whole cells incubated for short periods with mutanolysin. However, after continued incubation, the LFP band disappeared. This suggests that other lytic enzymes, presumably proteases, are also released by mutanolysin and that it is these enzymes that degrade the LFP.

Fimbrial structures from gram-negative organisms have generally been found to be composed of subunits which are held to each other by hydrophobic and ionic bonds (21). Fimbrial proteins from *Escherichia coli* are insoluble in their native form but may be disaggregated by guanidine hydrochloride (10) or HCl (31) into monomers with MWs of 17,000 and 19,000. However, if the LFP described here consists of subunits, then it seems likely that they are held together by covalent bonds, since none of the treatments (urea, guanidine, HCl, or sodium thiocyanate) produced subunits. Fimbrial proteins from the gram-positive organisms *Actinomyces viscosus* (48) and *S. salivarius* (46) have also proved resistant to dissociation into subunits, although this cannot be taken as a general rule for gram-positive organisms because fimbriae on *C. renale* were easily disaggregated.

**TABLE 2. Adhesion-blocking activity of F(ab′)2 fragments of purified polyclonal IgG**

<table>
<thead>
<tr>
<th>IgG source</th>
<th>Mean adhesion* to S-HA (10^9 cells/ml) ± SD</th>
<th>Relative adhesion (%)</th>
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<tbody>
<tr>
<td>Nonimmune</td>
<td>1.32 ± 0.06</td>
<td>100</td>
</tr>
<tr>
<td>Antifibril</td>
<td>0.20 ± 0.29</td>
<td>15</td>
</tr>
<tr>
<td>Anti-LFP</td>
<td>1.2 ± 0.01</td>
<td>91</td>
</tr>
<tr>
<td>HEPES buffer</td>
<td>1.49 ± 0.19</td>
<td>113</td>
</tr>
</tbody>
</table>

* 3 x 10^9 cells were added to 40 mg of S-HA.
gated (24). Donkersloot et al. (5) have recently succeeded in cloning a structural gene for \textit{A. viscosus} type 2 fimbriae into \textit{E. coli}, resulting in the expression of a 59-kilodalton fimbrial protein, suggesting a subunit composition for this structure.

The MW of our fimbrial protein is in the same range as the fimbrial antigens b and c of \textit{S. salivarius}, which have been identified as the \textit{Veillonella}-binding protein and host attachment factor, respectively (45, 47). The LFP also resembles the \textit{S. salivarius} antigens in containing low levels of basic amino acids, relatively high levels of nonpolar amino acids, and, as in the case of antigen C, a high percentage of carbohydrate. Contamination from peptidoglycan was considered unlikely to be a major factor affecting the amino acid analysis, since Rosan (42) has demonstrated that, unlike our fimbrial protein, cell walls of the majority of strains of \textit{S. sanguis} contain high levels of alanine and only small quantities of glycine.

The observation that adherence to S-HA is sensitive to antifibrillar antibody suggests that the fibril may be one of the S-HA adhesins. This conclusion is supported by the finding that the nonadhering strain 12L does not possess the fibrils (34).

Fimbriae have been reported to be involved in adhesion of \textit{S. sanguis} to S-HA (11, 13, 15). Gibbons et al. (15) found that nonfimbriated variants were nonadherent and hydrophilic, while Falchon-Kalweit et al. (11) were able to inhibit binding to S-HA with antibody raised against purified fimbriae.

The inability of anti-LFP antibody to inhibit binding could be a result of the loss of active-site antigenic determinants caused by treatment of the fimbrii with SDS prior to electrophoresis. Anti-LFP antibody was raised against LFP protein excised from a denaturing gel. Such a protein would probably not retain its tertiary structure and may have lost the epitopes required to induce formation of antibodies blocking adherence. Alternatively, the fibrils may contain more than one type of subunit and the binding site may be on a subunit distinct from the LFP. The finding that Mab 32.33 bound only to the terminus of fibrils supports the concept of a unique terminal subunit. Alternatively, the Mab may recognize a terminal epitope which is hidden by opposing subunits and therefore is only available at the terminus.

Fibrils could not be found in preparations made from strain 12na, the organism which was shown previously to lack one of the two adhesins responsible for binding of \textit{S. sanguis} 12 to S-HA (33). The model proposed to explain binding of this organism to S-HA postulated that there was an adhesin which reacted with a pH-sensitive salivary receptor and a second adhesin which reacted with a neuraminidase-sensitive receptor. Strain 12na lacked the adhesin recognizing the neuraminidase-sensitive receptor. Strain 12na lacked the adhesin recognizing the neuraminidase-sensitive receptor. Given the inability to isolate a fibril from strain 12na, it is reasonable to speculate that the LFP is the adhesin which recognizes the neuraminidase-sensitive receptor. Work is in progress to further characterize the chemical and functional nature of the fibrils and to identify other fimbrial structures on the surface of \textit{S. sanguis}.

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**LITERATURE CITED**


