Glucosyltransferase Mediates Adhesion of *Streptococcus gordonii* to Human Endothelial Cells In Vitro

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Human umbilical vein endothelial cells (HUVEC) were used as an experimental host model to investigate the mechanism(s) of streptococcal adhesion in infective endocarditis. Adhesion activity of *Streptococcus gordonii* was maximal during the logarithmic phase of growth and was greatly reduced or eliminated by pretreatment of bacteria with heat, formaldehyde, or trypsin. At saturating numbers of streptococci, an average of 81 bacteria were bound per HUVEC. Streptococcal adhesion was inhibited by low-molecular-weight dextran and heparin but not by sucrose, fibronectin, or laminin. Adhesion was also prevented by pretreatment of HUVEC with proteins dissociated from the surface of *S. gordonii* with 10 mM EDTA or isolated from spent culture medium. Western blot (immunoblot) assays detected a single adhesin protein of 153 kDa (AP153) on HUVEC after incubation with unfractionated extracts of streptococci. The adhesin exhibited glucosyltransferase (GTF) activity when incubated with sucrose and Triton X-100 after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The AP153 was purified by affinity chromatography on dextran beads and shown to have binding activity for HUVEC, GTF activity, an amino acid composition similar to that reported for GTF of *S. gordonii*, and the ability to inhibit *S. gordonii* adhesion. Incubation of the streptococci with antibodies to the adhesin inhibited bacterial attachment to HUVEC monolayers. These results indicate that surface-localized GTF mediates adhesion of *S. gordonii* to HUVEC in vitro and may serve as a mechanism for colonization of the endocardium in infective endocarditis.

Infective endocarditis is frequently caused by oral alpha-hemolytic streptococci, such as *Streptococcus sanguis*, *S. gordonii*, *S. mutans*, and *S. mitis* (1, 36, 44, 53). The streptococci can enter the blood through breaks in the microcirculation of oral tissues induced by trauma, including dental manipulations, oral hygiene procedures, mastication, and oral infections (31). Streptococci, circulating in blood, may then adhere to damaged endothelium (2, 17) or thrombi composed of platelets and fibrin (2, 10) on vessel walls, endocardium, and heart valves. Further development of thrombi and verrucous vegetations on valves serves to embed the streptococci and provide protection from host defenses (10, 52) and antibiotic therapy (5).

Several species of oral streptococci have been shown to adhere in vitro to human endocardial, endothelial, and buccal epithelial cells (40, 49) and to endothelial cells isolated from rat heart (41). This adhesion may be dependent on tissue-localized fibronectin, since *S. sanguis* has been reported to bind to a conformationally specific domain on the immobilized form of fibronectin (24, 25) which can occur on cell surfaces and underlying extracellular matrix. There is also evidence that these bacteria can adhere to laminin, another protein in the subendothelial matrix, which can be exposed by damage to endocardium (42, 45, 50). Platelets and fibrin also accumulate on collagen when the extracellular matrix is exposed, giving rise to nonbacterial thrombotic vegetations which can serve, in turn, as targets for streptococcal colonization. *S. sanguis* can

adhere to platelets (14) through the action of fibrillar cell wall protein(s), causing a mixed agglutination (15).

The mechanisms by which alpha-hemolytic streptococci initially adhere to cardiac tissue have not been defined. Surface glucans, synthesized by extracellular glucosyltransferase (GTF; EC2.4.1.5), have been implicated in the adhesion of *S. sanguis* to rabbit heart valves with preexisting nonbacterial thrombotic endocarditis (39) and in adhesion to damaged canine heart valves covered with fibrin and platelets (34). Electron microscopy has confirmed the production of exopolysaccharides (glycocalyx) by streptococci in valvular vegetations in infected rabbits (28). A role for glucans in adhesion is supported by the observation that low-molecular-weight dextran inhibits *S. sanguis* adhesion to damaged canine heart valves (35) and that pretreatment of dextran-producing streptococci with dextranase decreases their ability to cause experimental infective endocarditis (34). Studies of viridans group streptococci isolated from patient blood have shown that polysaccharide production correlates closely with their pathogenicity in endocarditis (6). An isogenic mutant of *S. mutans*, which differed from the wild-type strain in several genes involved in glucan and fructan syntheses, showed less virulence in experimental infective endocarditis in rats, more susceptibility to phagocytosis in vitro, and reduced ability to adhere to fibrin deposits in vitro (30). However, a similar study with the rat infection model showed that the colonizing activity of a GTF-deficient mutant of *S. gordonii* did not differ significantly from that of an isogenic parental strain (51a). Other investigators (4, 16) have reported that the ability of streptococci to colonize fibrin-platelet clots in vitro does not correlate closely with the ability to produce extracellular glucans in vitro.

In this report, we identify a 153-kDa protein on the surface of *S. gordonii* (previously *S. sanguis*) that mediates bacterial adhesion to human endothelial and epithelial cells in vitro. Purified adhesin has GTF activity, binds directly to host cells,

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and blocks subsequent adhesion of viable streptococci. We also show that adhesion of *S. gordonii* to endothelial cells can be inhibited with low-molecular-weight dextrans and antibodies specific to the 153-kDa protein.

MATERIALS AND METHODS

Bacteria. *S. gordonii* G9B (previously *S. sanguis* G9B; see reference 51), *S. gordonii* Challis, and *S. sanguis* 38 were obtained from B. Rosan, University of Pennsylvania, Philadelphia. *Escherichia coli* HB101 was obtained from D. Dyer, State University of New York at Buffalo, Buffalo. *S. gordonii* G9B, an oral isolate, was selected as the focus of this study because preliminary experiments indicated that it has strong binding activity for cultured epithelial cells along with its known affinity for salivary glycoproteins (23) and oral epithelial cells (9) and because this species is often associated with infective endocarditis (1, 44, 53). Bacteria were grown for 18 h in adenine-deficient chemically defined medium (46) supplemented with 20 μ Ci of 3 H-adenine (ICN Radiochemicals, Irvine, Calif.). At the time of harvest, the specific radioactivity of the bacteria was typically 31,000 cpm per 10^8 cells.

Animal cells. Human umbilical vein endothelial cells (HUVEC) were isolated by the method of Jaffee et al. (20) and cultured in medium 199 (GIBCO, Grand Island, N.Y.) supplemented (per milliliter) with 500 U of penicillin G, 5 U of streptomycin sulfate, 5 μ g of amphotericin B, 20% fetal bovine serum (GIBCO), 7.5 μ g of endothelial cell growth supplement, 5 U of heparin (Sigma Chemical Co., St. Louis, Mo.), 5 μ l of minimal essential medium amino acids (GIBCO) and 10 μ l of minimal essential medium vitamins (GIBCO). The monolayers were grown in 48-well plastic plates for the bacterial adhesion assays. Microscopic morphology of the cells and their reactivity with monoclonal antibodies to human factor VIII (Accurate Chemical and Scientific, Westbury, N.Y.) (19) indicated that the monolayers contained >95% endothelial cells.

Buccal epithelial cells were collected by gentle scraping of the buccal mucosa of a blood type B(Rh⁻) female donor. The cells were washed three times in 10 ml of 0.15 M sodium chloride–0.01 M sodium phosphate at pH 7.2 (phosphate-buffered saline [PBS]) by centrifugation at $1,250 \times g$ for 15 min and were then suspended, using a glass tissue homogenizer (Inframo, Wayne, N.J.). HEP-2 cells were obtained from the American Tissue Type Culture Collection (CCL 23) and were grown on GIBCO medium 199 supplemented with 10% fetal bovine serum and antibiotics under air with 5% CO₂. Sheep erythrocytes were isolated from citrated whole blood by centrifugation at $1,250 \times g$ for 15 min. The cell pellet was washed twice in PBS and resuspended to 1.8×10^7 cells per ml.

Bacterial adhesion assay. Adhesion of *S. gordonii* to endothelial cell monolayers was measured by using a modification of the bacterial invasion assay of Isberg and Falkow (18). Radiolabeled bacteria were suspended in GIBCO medium 199 to a density of 10^9 cells per ml and added to monolayers of endothelial cells in 48-well plates. In most assays, 2.5×10^7 bacteria were used per well. The bacteria were sedimented onto the monolayer by centrifugation at $175 \times g$ for 10 min to accelerate the kinetics of cell contact. The nonadherent bacteria were removed by washing the monolayer three times in PBS for 1 min each time. The adherent bacteria were recovered by extraction of the monolayer with 200 μ l of 2% sodium dodecyl sulfate (SDS) and counted by liquid scintillation spectrometry. Bacteria were also incubated in wells containing only tissue culture medium as a negative control. The total numbers of adherent streptococci were calculated from the specific radioactivity of the original bacterial suspension. The

data are expressed as the number of streptococci per HUVEC. The average number of HUVEC per monolayer (2.51×10^5) was determined by dissociating the cells with trypsin and counting them microscopically in a hemocytometer.

To determine binding specificity of streptococcal adhesins, bacteria were assayed for adhesion activity in the presence of potential competitive inhibitors: mono- and disaccharides, dextran (10,000 molecular weight), laminin, fibronectin, heparin, heparan sulfate, and chondroitin sulfate (Sigma). In some experiments, the HUVEC were preincubated for 1 h at 37°C with the indicated amounts of streptococcal components to block adhesion receptors. Subsequent adhesion of bacteria was normalized to the amount of adhesion to untreated monolayers. In experiments to define the effects of antibodies on adhesion activity, 2.5×10^7 streptococci were incubated for 18 h at 4°C with selected dilutions of normal rabbit serum, antiserum to *S. gordonii* G9B, rabbit immunoglobulin G (IgG) antibodies to *S. gordonii* G9B, or affinity-purified antibodies to the 153-kDa protein. The bacteria were washed three times by centrifugation at $1,250 \times g$ for 15 min with 10 ml of medium 199 and tested in the endothelial cell adhesion assay as described above. Adhesion activity was normalized to the amount obtained with untreated streptococci.

Extraction of bacteria. Ten grams of moist *S. gordonii* G9B cells was suspended in 100 ml of 0.1 M Tris-HCl (pH 8.0)–10 mM EDTA–1 mM phenylmethylsulfonyl fluoride (Sigma), and the suspension was stirred for 18 h at 4°C. After the bacteria were pelleted by centrifugation at $12,500 \times g$ for 45 min, the supernatant fluid was passed through a 0.45- μ m-pore-size polycarbonate filter (Millipore, Bedford, Mass.) and dialyzed against water for 3 days with six changes. The retentate was lyophilized and stored desiccated at room temperature. The protein yield was 30 mg from 10 g of moist packed cells.

Bacterial proteins released by the streptococci during growth were recovered from the culture medium by ultrafiltration on a tangential flow apparatus (10,000-molecular-weight pores; Millipore). The concentrate was supplemented with 1 mM phenylmethylsulfonyl fluoride and then dialyzed against water and lyophilized. An average of 10.2 mg of streptococcal protein was recovered from 1 liter of culture.

Chemical analyses. Carbohydrate was quantified by the phenol-sulfuric acid procedure of Dubois et al. (7), using glucose as the standard. Protein concentrations were determined by the method of Hartree (13), using bovine serum albumin as a standard. Amino acid composition was determined after hydrolysis of samples in 6 N HCl for 24 h at 110°C. Samples were analyzed in an amino acid analyzer (model 6300A; Beckman Instruments, Inc., Fullerton, Calif.) (32).

Electrophoresis. SDS-polyacrylamide gel electrophoresis (PAGE) was performed in 7.5% acrylamide slab gels (22). The broad-range molecular weight protein kit (Bio-Rad) was used for standards, which were detected by staining with silver (29). Western blot (immunoblot) assays were performed with rabbit antisera to whole *S. gordonii* or to EDTA-extracted components of *S. gordonii* and alkaline phosphatase-conjugated goat antibodies to rabbit IgG (47).

Trypsinization of streptococci. Streptococci were suspended at 10^9 cells per ml in 0.5 mM EDTA–0.1% trypsin (GIBCO) for 1 h at 30°C. The bacteria were collected by centrifugation, washed three times with 50 ml of PBS, and tested in the adhesion assay. In other experiments, the trypsinized streptococci were extracted with NaOH at pH 11, and the solubilized components were analyzed by SDS-PAGE and silver stain.

EDTA-solubilized streptococcal components (50 μ g of protein) were treated with 10 U of trypsin cross-linked to agarose beads (Sigma) at 37°C for 1 h. After removal of the trypsin-

agarose beads by centrifugation, the supernatant fluid was tested in the indicated assays.

Adhesin assay. To identify the streptococcal adhesin(s), 1.8×10^6 host cells were suspended in 0.1 ml of medium 199 containing 100 μ g of unfractionated streptococcal protein. After 1 h at 22°C, the cells were harvested by centrifugation at $1,250 \times g$ for 15 min, washed three times with 200 μ l of medium 199, and transferred to a clean vial. The treated cell pellets were then solubilized in 1% SDS. The solubilized cell constituents were resolved by SDS-PAGE and identified by Western blot assay, using rabbit antiserum to *S. gordonii*.

GTF assay. Streptococcal proteins were resolved by SDS-PAGE and tested for GTF activity by the method of Russell (37). Gels containing 50 μ g of protein were incubated in 200 mM sucrose–1% sodium azide–1% Triton X-100–50 mM sodium acetate (pH 5.5) for 48 h at 37°C. In some experiments, dextran 10,000 was included as a primer for glucan synthesis. The soluble reactants were removed by soaking the gel in acetate buffer for 48 h. This step was followed by incubation in 1.0% periodic acid–7% acetic acid for 1 h at 4°C. The gels were then stained for glucan by periodic acid-Schiff (PAS) reagent (21). For controls, polyacrylamide gels containing identical amounts of streptococcal proteins were treated with sucrose-free solution and processed in the same manner.

Adhesin purification. The 153-kDa adhesin protein (AP153) was purified from cell extracts and spent culture medium by affinity chromatography on dextran beads, using a modification of the method of McCabe (27). Ten milligrams of unfractionated extracellular streptococcal protein in 10 ml of 0.02% NaN_3 was loaded onto a column (5 by 1 cm) of Sephadex G-50. After unbound proteins were eluted from the column with 100 ml of 0.02% NaN_3 , the 153-kDa protein was eluted with 25 ml of 6 M guanidine hydrochloride. The protein solution was dialyzed against water, quick-frozen on dry ice, and lyophilized. The yield of AP153 was 0.1 mg from 10 mg of unfractionated protein.

In later experiments, lyophilized crude streptococcal proteins were dissolved at 10 mg/ml in 0.15 M NaCl–0.01 M EDTA–0.05% SDS–0.01 M Tris-acetate (pH 7). After removal of insoluble material by centrifugation at $30,000 \times g$ Triton X-100 was added to 0.5% (by volume). The solution was loaded onto a column (1 by 5.5 cm) of Sephadex G-50 equilibrated with 0.5% Triton X-100–0.01 M EDTA–0.15 M NaCl. The column was eluted with 100 ml of column buffer minus Triton X-100. The adsorbed protein was eluted from the column with 8 M urea as a single peak absorbing at 280 nm. The protein was dialyzed against water, quick-frozen on dry ice, and lyophilized for storage. The yield of AP153 with this procedure was 0.5 mg from 10 mg of unfractionated extracted protein.

Antiserum production. New Zealand White rabbits were immunized intramuscularly with 2 mg of EDTA-extracted streptococcal proteins, 2 mg of medium-derived streptococcal proteins, or 2×10^8 freeze-thawed *S. gordonii* G9B cells in incomplete Freund's adjuvant. The animals received booster injections at 2, 4, and 6 weeks. The rabbits were anesthetized and exsanguinated between weeks 8 and 10. The resulting sera were heated at 60°C for 30 min and stored at –20°C. Antibodies to the 153-kDa adhesin were affinity purified on antigen-coated nitrocellulose paper by the method of Maniatis et al. (26).

Enzyme immunoassay. Endothelial cell monolayers, in 48-well culture plates, were washed three times for 1 min each at room temperature with 200 μ l of GIBCO medium 199 and then incubated for 1 h at 37°C with 200 μ l of medium 199 containing 0 to 50 μ g of *S. gordonii* G9B protein. After

FIG. 1. Adhesion activity of bacteria harvested during logarithmic (□), early stationary (●), and late stationary (■) phases of growth. Data represent the averages $\pm$ standard deviations of duplicate assays.

unbound reactants were removed by washing three times with medium 199, the monolayers were incubated with 200 μ l of rabbit antiserum to *S. gordonii* for 1 h at 37°C. Bound rabbit antibodies were measured spectrophotometrically (405 nm) after incubation with alkaline phosphatase-conjugated goat antibodies to rabbit IgG and disodium *p*-nitrophenylphosphate. All assays were performed in duplicate. Controls consisted of endothelial cell monolayers incubated with only the primary and secondary antibody reagents.

RESULTS

Streptococcal adhesion. *S. gordonii* G9B cells were harvested in the logarithmic growth phase (1 h before maximum growth) and at early and late stationary phases (4 and 8 h after maximum growth, respectively) and incubated with endothelial cell monolayers. Figure 1 shows that most adhesion was obtained with logarithmic cells; there were 85 bacteria bound per HUVEC in monolayers incubated with 5×10^7 streptococci. The adhesion activity of *S. gordonii* decreased sharply with the age of the culture. When HUVEC were incubated with 1.25×10^7 streptococci, a subsaturating number (76% of logarithmic-phase bacteria, 53% of early-stationary-phase bacteria, and 13% of late-stationary-phase bacteria) adhered. Microscopic analysis of the initial bacterial suspensions indicated that the differences in the amount of adhesion were not due to variations in the number of cells in the streptococcal chains. Chain lengths ($\pm$ standard deviation) were 2.03 ± 0.55 , 2.23 ± 0.52 , and 2.79 ± 1.2 , respectively. Additional experiments with saturating numbers of logarithmic-phase streptococci indicated that each endothelial cell bound 81 ± 10 (standard deviation) streptococci. Microscopic analysis showed that the bacteria were located randomly over the exposed surfaces of HUVEC. In comparison, *S. gordonii* Challis, *S. sanguis* 38, and *E. coli* HB101 were 105, 126, and 19% as adherent to HUVEC as *S. gordonii* G9B, respectively.

The ability of *S. gordonii* to adhere was reduced sharply by pretreatment with heat, formaldehyde, and proteases (Table 1), indicating that bacterial surface proteins played a prominent role in the adhesion mechanism. Pretreatment of the bacteria with 10 mM EDTA reduced adhesion activity by 49%, which could not be restored by addition of 1 mM Ca^{2+} or

TABLE 1. Effects of physical and chemical treatment of streptococci on adhesion to HUVEC

Treatment	Adhesion (% $\pm$ SD) ^a
None.....	100
60°C, 15 min.....	66 $\pm$ 4.8
80°C, 15 min.....	22 $\pm$ 0.7
Formaldehyde, 2%.....	10 $\pm$ 1.0
Trypsin, 0.1%.....	1 $\pm$ 0.1
EDTA, 10 mM.....	49 $\pm$ 10.0

^a The amounts of adhesion obtained with treated bacteria in duplicate assays were compared with those of untreated bacteria (control). The adhesion activity of untreated streptococci was 52 $\pm$ 1.2 bacteria per HUVEC.

Mg²⁺. Analysis of the supernatant fluid indicated that EDTA solubilized 3 mg of protein per g of moist packed cells. The bacteria remained intact, as determined by phase-contrast and electron microscopy (data not shown); however, the number of CFU was reduced by 72%. The proteins extracted from *S. gordonii* appeared to be associated with the cell surface since pretreatment of the cells with trypsin reduced the amount of EDTA- or alkali (pH 11)-extractable protein by >95%.

Adhesin identification. The specificity of adhesion was studied by inhibition assays (Table 2). The most effective complex inhibitors were dextran (70%) and heparin (55%). Weak inhibition (<25%) was observed with the extracellular matrix components: laminin, fibronectin, heparan sulfate, and chondroitin sulfate. Of the mono- and disaccharides tested, only *N*-acetylneuraminic acid and glucuronic acid were inhibitory, suggesting that anions had a nonspecific inhibitory effect.

Pretreatment of endothelial cell monolayers with 100 μ g of EDTA-extracted components of *S. gordonii* or with 100 μ g of streptococcal components isolated from spent culture medium inhibited subsequent adhesion of viable streptococci by 60% $\pm$ 10% and 40% $\pm$ 8%, respectively. The blocking activities of the bacterial components were completely eliminated by heating at 80°C and by digestion with trypsin-agarose beads. Figure 2 shows a dose-response binding curve obtained with isolated streptococcal components and endothelial cells in an enzyme immunoassay. Comparison of extracts of streptococci, from different points in the growth curve, showed that binding activity of streptococcal components was maximal in logarithmic-phase bacteria. The relative activity of these extracts was

FIG. 2. Binding of soluble components extracted from *S. gordonii* to HUVEC monolayers in an enzyme immunoassay with rabbit antiserum to whole *S. gordonii*. Symbols: $\square$, EDTA extract of mid-logarithmic-phase bacteria; $\bullet$, EDTA extract of late-stationary-phase bacteria; $\blacksquare$, bacterial proteins from culture medium 4 h after maximum growth. Data represent averages $\pm$ standard deviations of duplicate assays.

consistent with the observation that adhesion activity of intact bacteria was greatest during logarithmic growth.

To identify the individual adhesin(s), EDTA-extracted components were incubated with suspended endothelial cells which were subsequently tested for bound streptococcal components by Western blot assay. A single band, migrating at 153 $\pm$ 3 kDa, was observed in extracts of treated HUVEC (Fig. 3). The binding activity of this streptococcal component was inactivated by pretreatment of the bacterial extract at 80°C for 15 min or by trypsin digestion, as was the adhesion activity of the bacterium. The 153-kDa adhesin protein (AP153) also bound in vitro to human buccal epithelial cells and to HEp-2 cells but not to sheep erythrocytes (Fig. 4). In other experiments, the streptococcal proteins did not show binding activity for laminin or fibronectin that was adsorbed to polyvinyl microtiter wells in an enzyme immunoassay.

TABLE 2. Competitive inhibition of streptococcal adhesion to HUVEC

Inhibitor	Concn	Adhesion (% $\pm$ SD) ^a
Glucose	50 mM	98 $\pm$ 11
Glucuronic acid	50 mM	60 $\pm$ 3
<i>N</i> -Acetylneuraminic acid	50 mM	65 $\pm$ 5
Sucrose	50 mM	90 $\pm$ 5
Dextran 10,000	1 mM	30 $\pm$ 2
Chondroitin sulfate	1%	80 $\pm$ 2
Heparan sulfate	1%	87 $\pm$ 8
Heparin	1%	45 $\pm$ 5
Fibronectin	1%	80 $\pm$ 2
Laminin	1%	75 $\pm$ 2

^a The amounts of adhesion observed with treated bacteria in duplicate assays were averaged and recorded as a percentage of the activity of untreated positive control cells (57 $\pm$ 3 bacteria per HUVEC). Other carbohydrates tested (fucose, galactose, mannose, *N*-acetylglucosamine, ribose, xylose, and lactose) inhibited less than 5% adhesion.

FIG. 3. Identification of streptococcal adhesin by Western blot assay, using antiserum to whole *S. gordonii* cells. Lanes: 1, 10 μ g of EDTA-extracted proteins from logarithmic-phase bacteria; 2, extract of 1.8×10^6 HUVEC pretreated with 10 μ g of EDTA-extracted components of logarithmic-growth-phase bacteria; 3, extract of untreated 1.8×10^6 HUVEC; 4, 10 μ g of culture medium-derived streptococcal proteins; 5, extract of 1.8×10^6 HUVEC pretreated with 10 μ g of medium-derived streptococcal components.

FIG. 4. Western blot analyses of the binding activity of the 153-kDa streptococcal protein to various types of mammalian cells. Lanes: 1, 10 μ g of EDTA-extracted streptococcal protein; 2, extract of 1.8×10^6 HEp-2 cells pretreated with EDTA-extracted streptococcal components; 3, extract of 1.8×10^6 untreated HEp-2 cells; 4, extract of 1.8×10^6 buccal epithelial cells pretreated with EDTA-extracted streptococcal components; 5, extract of untreated buccal epithelial cells; 6, extract of 1.8×10^6 sheep erythrocytes pretreated with EDTA-extracted streptococcal components. Streptococcal components were detected with rabbit antiserum to whole *S. gordonii* cells.

Purification and characterization of the adhesin. AP153 was affinity purified from spent culture medium (4 h after maximum growth) with a column of Sephadex G-50 and constituted approximately 1% of the total protein in the initial unfractionated preparation. Culture medium was selected for isolation of AP153 because of its easy accessibility and lower content of contaminating proteins. Purity was verified by SDS-PAGE (silver stain) and by Western immunoblots, using antiserum to *S. gordonii* (Fig. 5). Because the molecular mass of the isolated *S. gordonii* protein was similar to that of a GTF reported in *S. gordonii* (12), the protein was tested for enzyme activity in an SDS-PAGE zymogram. PAS staining indicated that glucan was formed from sucrose by the AP153 in unfractionated extracts of logarithmic- and stationary-phase cells and in protein purified from spent culture medium (Fig. 6). Its amino acid content is shown in Table 3 and is similar to that previously reported

FIG. 5. PAGE analysis of purity and enzymatic activity of the 153-kDa protein of *S. gordonii*. Lanes: 1, silver stain of 20 μ g of EDTA-extracted proteins; 2, silver stain of 20 μ g of culture medium-derived streptococcal proteins; 3, silver stain of 2 μ g of purified adhesin protein; 4, Western blot assay of 7.5 μ g of purified adhesin protein reacted with antiserum to whole bacteria; 5, Western blot assay of an extract of 1.8×10^6 HUVEC that were preincubated with 2 μ g of purified 153-kDa adhesin protein; 6, PAS stain of 2 μ g of purified 153-kDa protein after incubation with sucrose.

FIG. 6. PAS-stained polyacrylamide gel showing location of polysaccharide synthesis following incubation with sucrose. Lanes: 1, 50 μ g of EDTA-extracted protein of logarithmic-phase streptococci; 2, same load as lane 1 but not incubated with sucrose; 3, 50 μ g of EDTA-extracted protein from late-stationary-phase streptococci; 4, 50 μ g of streptococcal protein obtained from culture medium at early stationary phase of growth.

for a 156-kDa GTF in *S. gordonii* (12). No amino sugars were detected in the protein hydrolysate. The protein was not stained by PAS reagent nor was carbohydrate detected in a phenol-sulfuric acid assay (<1% of dry weight).

The role of AP153 as a bacterial adhesin was also indicated by the ability of the purified protein to block bacterial adhesion to endothelial cells. Maximum inhibition of $40\% \pm 5\%$ was attained at 12.5 μ g of AP153 per 2.5×10^5 endothelial cells. Higher concentrations of AP153 did not cause additional inhibition, possibly because of autoaggregation of the protein and reassociation of the protein with the bacterial surface or because of other possible adhesins. Similar amounts of bovine serum albumin and heat-inactivated AP153 caused no inhibition of adhesion. Antibodies specific for AP153 inhibited adhesion of viable streptococci (Table 4). Bacteria pretreated with unfractionated antiserum to *S. gordonii*, IgG purified from this serum, and affinity-purified antibodies to AP153 lost 92, 82, and 60% of their adherence activity, respectively. Serum from nonimmunized rabbits did not inhibit adhesion in these experiments. Antibodies to AP153 were observed to bind to

TABLE 3. Amino acid composition of the 153-kDa adhesin protein

Amino acid	Residues/100 residues	
	AP153	GTF156 ^a
Aspartate or asparagine	13.0	16.1
Threonine	6.8	6.3
Serine	6.5	5.2
Glutamate or glutamine	12.3	11.3
Proline	3.2	2.2
Glycine	14.6	8.6
Alanine	10.7	7.6
Half-cystine	0.0	0.0
Valine	5.6	5.6
Methionine	0.8	1.9
Isoleucine	3.9	3.3
Leucine	6.5	6.7
Tyrosine	3.3	6.1
Phenylalanine	5.5	4.0
Histidine	0.6	0.8
Lysine	3.8	7.9
Arginine	3.0	3.9
Tryptophan	ND ^b	2.3

^a Data obtained from Grahame and Mayer (12).

^b ND, not determined.

TABLE 4. Effect of antibodies on streptococcal adhesion to HUVEC

Antibody	Amt	Adhesion (% $\pm$ SD) ^a
Control		100 $\pm$ 8
Normal serum	1:100	140 $\pm$ 3
Antiserum to whole bacteria	1:100	8 $\pm$ 1
IgG to whole bacteria	1.5 μ g	18 $\pm$ 1
Affinity-purified IgG to 153-kDa adhesion	1.5 μ g	40 $\pm$ 2

^a Bacteria (2.5×10^7) were incubated for 18 h at 4°C with rabbit sera or purified antibodies prior to the adhesion assay. The amounts of adhesion shown by treated streptococci in duplicate assays were compared with those observed with untreated bacteria (positive control). The adherence activity of the untreated streptococci was 65.2 ± 5 bacteria per HUVEC.

the surfaces of *S. gordonii* G9B in a dot blot immunoassay and an indirect immunofluorescence assay, confirming its surface location (data not shown).

DISCUSSION

The evidence presented here demonstrates that a surface-localized, 153-kDa protein with GTF activity mediates the adhesion of *S. gordonii* to human endothelial cells in vitro. The adhesin and numerous other proteins can be dissociated from the cell wall by extraction with 10 mM EDTA without inducing cell lysis. Streptococci that were pretreated with trypsin, which removes fibrillar surface proteins (15, 16), did not release AP153 or other proteins upon extraction with EDTA or NaOH at pH 11. Interestingly, EDTA has been reported to prevent spontaneous aggregation of extracellular GTF of *S. gordonii* (11). These observations indicate that divalent cations (Mg^{2+} and Ca^{2+}) cross-link and stabilize surface proteins of *S. gordonii*. The effects of EDTA on the stability of gram-negative cell walls is also well documented (48). The sensitivity of adhesion activity of intact streptococci to inactivation by heat, formaldehyde, and protease treatments underscores the role of proteins but also indicates that surface-localized polysaccharides and lipoteichoic acid may not play major roles in the adhesion mechanism in this host model because these cell constituents are typically resistant. Furthermore, growth of *S. gordonii* in chemically defined medium devoid of sucrose eliminates the possibility of glucan biosynthesis and any subsequent interaction with HUVEC surfaces. It should also be emphasized that purified AP153 did not contain associated polysaccharide as indicated by PAS stain or by direct chemical analysis.

Purified AP153 bound directly to endothelial cells and blocked 40% adhesion of intact streptococci. The failure to completely inhibit bacterial adhesion may be explained by the ability of the adhesin on the endothelial cell to reattach to the bacterium and bridge the opposing cell surfaces. GTF of *S. gordonii* can form complexes with itself (11) and with surface glucan (11, 27, 51). Alternatively, other possible adhesins may contribute to the bacterial colonization mechanism; however, none were detected in the bacterial extracts prepared in this study. We detected only one size of GTF in extracts of *S. gordonii*, although a 170- to 180-kDa enzyme was often present in small amounts in culture medium preparations. Grahame and Mayer (12) found that a 174-kDa GTF is converted to a 156-kDa GTF by a bacterial protease which removes 177 amino acids from the secreted protein without altering the enzymatic properties of the GTF. Both forms of GTF are bifunctional, producing glucans with 83% α -D-(1-6) and 17% α -D-(1-3) bonds independently of glucan primer. It is possible

that growth of streptococci in chemically defined medium in the present study may result in increased protease (43) activity which converts most of the GTF174 to GTF156 (AP153). The addition of a protease inhibitor, phenylmethylsulfonyl fluoride, iodoacetic acid, EDTA, or *N*-tosyl-L-phenylalanine chloromethyl ketone, to the extracted proteins does not diminish the proteolytic conversion (11; this study). In addition to the enzymatic activity and molecular weight, the chemical composition of the purified AP153 was similar to that reported for GTF156 of *S. gordonii* 10558 (12). The production of AP153 by *S. gordonii* during growth is also similar to that previously observed for GTF156, in which maximum levels of activity were attained during the logarithmic phase of growth and diminished steadily during the stationary growth phase. We found that bacterial adhesion activity decreased 79% within 8 h after maximum growth in chemically defined medium, and Buchan and Jenkinson (3) found that 90% of GTF activity is lost under similar growth conditions.

The ability of GTF to function as an adhesin is consistent with earlier reports that the dextran-producing ability of viridans group streptococci correlates closely with their virulence in experimental infective endocarditis (10, 30, 34, 35). Teleologically, the ability of GTF to exist as high-molecular-weight homogeneous complexes (11) or as heterologous complexes with polysaccharides (51) would enable it to assume a prominent superficial cellular location where it can readily make contact with host surfaces. As polysaccharide accumulates on the bacterial surface in a sucrose-containing environment, GTF would be expected to remain associated with the outer boundaries of the glycocalyx, whereas fibrillar proteins that are anchored to the cell wall matrix, analogous to M protein of group A streptococci (33), might be concealed by the polysaccharide. The GTF is not expected to contribute to glycocalyx synthesis by bacteria growing in endocardial sites since sucrose is not present in blood (10). However, oral streptococci entering the bloodstream with a preexisting glucan capsule are expected to avoid clearance by phagocytes and thus have a longer opportunity to colonize endocardial surfaces (30). A major role of AP153 (GTF) in the adhesion of *S. gordonii* to HUVEC is not necessarily inconsistent with the previous report by Herzberg et al. (16), who found no correlation between the production of extracellular GTF and the ability of streptococci to adhere to platelets and to induce their aggregation in vitro. It is not yet known whether GTF also mediates streptococcal adhesion to platelets or what the relative amounts of cell-associated GTF are on each bacterial strain. Most importantly, our studies indicate that the relative age of the bacterial cultures must be considered in quantitative analyses of both extracellular and cell-associated GTF. Both bacterial adhesion and enzymatic activities decrease rapidly during the stationary phase of growth. These observations may also reconcile the disparate findings of Wells et al. (51a), namely, that a GTF-deficient mutant colonized heart valves as efficiently as an isogenic wild-type strain in a rat infection model. Because nonbacterial thrombi were artificially induced in the animals by a ventricular catheter prior to injection of bacteria, it is not known whether circulating streptococci adhered initially to endocardial cells and acted as a nidus for further thrombus formation or became lodged in an already developing platelet/fibrin mass.

The serological experiments show that streptococcal adhesion to endothelial cells in vitro is effectively blocked by antibodies to AP153 and indicate that immunization with this antigen might offer protection to infective endocarditis by *S. gordonii* and *S. sanguis*. Other studies (8, 38) have shown that immunization with killed *S. sanguis* or *S. mutans* can offer

protection against experimental infective endocarditis by these bacteria. The use of GTF vaccines, however, may require inclusion of enzymes from several *Streptococcus* species to ensure broad protection; for example, Buchan and Jenkinson (3) reported that antibodies raised to GTF of *S. gordonii* Challis also reacted with *S. sanguis* FW 227 and *S. oralis* (ATCC 10557) but not with *S. anginosus*, *S. mutans* (NCTC 10449), *S. salivarius*, or *S. sobrinus* 6715. Future studies should examine the possible role of AP153 (GTF) in the adhesion of *S. sanguis* to platelets and to nonbacterial thrombotic vegetations and whether specific antibodies are protective for experimental infective endocarditis by oral streptococci. Also, identification and characterization of the host cell components that bind streptococcal GTF will further our understanding of the mechanisms of streptococcal pathogenesis in infective endocarditis and may provide insight for the development of improved methods for prophylaxis or therapy.

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