Platelet-Interactive Products of *Streptococcus sanguis* Protoplasts

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To isolate a more native, platelet-interactive macromolecule (class II antigen) of *Streptococcus sanguis*, cultured protoplasts were used as a source. Protoplasts were optimally prepared from fresh washed cells by digestion with 80 U of mutanolysin per ml for 75 min at 37°C while osmotically stabilized in 26% (wt/vol) raffinose. Osmotically stabilized forms were surrounded by a 9-nm bilaminar membrane, as shown by transmission electron microscopy. Protoplasts were cultured in chemically defined synthetic medium and osmotically stabilized by ammonium chloride. Spent culture media were harvested daily for 7 days. Each day, soluble proteins were isolated from media, preincubated with platelet-rich plasma, and tested for inhibition of platelet aggregation induced by *S. sanguis* cells. Products released from *S. sanguis* protoplasts and reactive with an anti-class II antigen immunoaffinity matrix were able to inhibit *S. sanguis*-induced platelet aggregation. As resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, anti-class II-reactive protoplast products included silver-stained bands of 67, 79, 115, 216, and 248 kDa. The 115-kDa protein fraction was isolated by gel filtration and ion-exchange chromatography. This form of the class II antigen contained *N*-formylmethionine at its amino terminus. Rhamnose constituted 18.2% of the total residual dry weight and nearly half of its carbohydrate content. Diester phosphorus constituted 1% of this fraction. After trypsinization of the protoplast products from either preparation, a 65-kDa protein fragment was recovered. This protoplast protein fragment and the *S. sanguis* cell-derived 65-kDa class II antigen, previously implicated in the induction of platelet aggregation, were shown to be functionally and immunologically identical.

Certain strains of *Streptococcus sanguis* interact with platelets in vitro, triggering aggregation into thrombuslike clots (15, 16, 18). At least two different *S. sanguis* surface antigens mediate interactions with platelets. The class I antigen is an adhesin which binds *S. sanguis* cells to platelets. The class II antigen is isolated after trypsinization of *S. sanguis* cells (16) and purified by immunoaffinity chromatography or gel filtration and ion-exchange chromatography (9a). In this form, the class II antigen reacts with platelets in plasma to inhibit *S. sanguis*-induced platelet aggregation. This single-chain monomer contains about 90% protein and has an apparent molecular mass of 65 kDa. After the *S. sanguis* cell has attached, the native cell-associated class II antigen triggers human platelets to aggregate. The relationship of the soluble class II antigen released from the wall by trypsinization to a native cell wall-bound antigen is unclear.

We sought to develop a method to isolate a more native form of the *S. sanguis* class II antigen. For nearly 30 years, it has been known that streptococcus protoplasts will synthesize and export macromolecular substances (12, 22). When cultivated in liquid medium, streptococcus protoplasts will maintain balanced macromolecular biosynthesis (28). In these conditions, group A streptococcus protoplasts released a higher molecular weight form of M protein than was obtained by pepsin treatment of intact cells (36).

While *S. sanguis* is generally resistant to the muralytic effects of lysozyme, mutanolysin (a muramidase isolated from *Streptomyces globisporus*) has been used to produce protoplasts of several species of related streptococci, including *Streptococcus mutans* (26, 30), group B streptococci (4, 37), and *Streptococcus mitis* (20). These protoplasts dis-

played minimal leakage of cytoplasmic contents and were essentially free of cell wall products. In our study, *S. sanguis* cells were digested with mutanolysin to form protoplasts, which were cultured in osmotically stabilized, chemically defined synthetic medium. Platelet-interactive macromolecular class II antigen was recovered and characterized.

MATERIALS AND METHODS

Microorganisms: growth and preparation. The principal microorganisms used in this study, *Streptococcus sanguis* I 133-79 and I 2017-78, have been previously described as prototypic strains that are able to adhere to platelets and, in plasma, induce them to aggregate (15, 16, 18). Both strains are biotyped (11) blood culture isolates from confirmed cases of bacterial endocarditis provided by R. R. Facklam, Centers for Disease Control, Atlanta, Ga. Except when used to provide protoplasts, the bacteria were grown overnight at 37°C in Todd-Hewitt broth in 5 to 10% CO₂ (GasPak; BBL Microbiology Systems, Cockeysville, Md.), harvested by centrifugation (7,500 × *g*, 20 min, 4°C), and washed twice in cold 0.01 M sodium phosphate buffer, pH 7.0, with 0.9% (wt/vol) sodium chloride (PBS). After washing, the bacteria were dispersed and suspended to a final A₆₂₀ of 2.0 (4 × 10⁹ cells per ml) in PBS precisely as described in previous reports (15, 16, 18).

Protoplasts: preparation and culture. Whole *S. sanguis* cells required for protoplast conversions were initially grown in the liquid chemically defined synthetic medium (FMC) of Terleckyj et al. (33). FMC-grown cells were routinely harvested after 18 h of growth by centrifugation (7,500 × *g*, 20 min, room temperature [RT]). Cells were washed once in 0.05 M PBS, pH 6.8, twice in 0.005 M disodium-ethylene-diaminetetraacetic acid (Na₂EDTA), pH 6.8, and once in distilled water. The cells were resuspended to an optical

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density of 2.0 at 620 nm in 0.02 M Tris hydrochloride, pH 6.8, with 0.01 M $MgCl_2$ and 26% (wt/vol) raffinose. By using conditions that were determined experimentally to be optimal, mutanolysin (Sigma Chemical Co., St. Louis, Mo.) was added to cells at a final concentration of 80 U/ml and incubated for 75 min at 37°C. Proteolytic contamination of the mutanolysin was determined to be less than 0.25% when assayed with Azocoll as described by Siegel et al. (30). By 45 min, dilution of digested cells (1:5) into distilled water (dH_2O) resulted in a maximal loss of turbidity, but apparent release of the peptidoglycan and polysaccharide markers (26), muramic acid (13), and rhamnose (8) was not maximized until 75 min of digestion. At 75 min, 0.6% of digested cells were viable after growth and enumeration on nutrient agar spread plates.

Prior to culture, protoplasts were isolated from the digest by sedimentation at $15,000 \times g$ for 30 min at RT and resuspended in FMC with 0.019 M Na_2CO_3 and 0.5 M ammonium chloride to inactivate the mutanolysin and provide osmotic stability. Whereas raffinose was now omitted, penicillin (5 $\mu g/ml$) was added to inhibit cell wall synthesis (26, 35). The ability to generate *S. sanguis* protoplasts was routinely evaluated by the appearance of refractive "soap bubble" forms in phase-contrast microscopy and by osmotic fragility in dH_2O . Every 24 h, the protoplasts were sedimented ($15,000 \times g$, 30 min, RT) and resuspended in fresh medium. The spent medium was saved, dialyzed at 4°C (20 volumes of 0.2% Na_2EDTA followed by 40 volumes of dH_2O), lyophilized, and analyzed for total soluble protein by the method of Lowry et al. (21).

Electron microscopy. Transmission electron microscopy was used to examine both whole cells and protoplasts by modifications of techniques reported previously (15, 16). *S. sanguis* cells were resuspended after washing to approximately $4 \times 10^9/ml$ in Tris-raffinose buffer and warmed to 37°C. After the addition of mutanolysin (80 U/ml) to 10 ml of bacterial suspension, incubation was continued at 37°C for 75 min. A second aliquot was treated in the same manner, without the addition of enzyme. Cells were then centrifuged at $15,000 \times g$ for 30 min, washed once in Tris-raffinose buffer, pelleted, and fixed first with cold 0.2% glutaraldehyde in Hank's balanced salt solution, pH 7.3, for 10 min. After the solution was removed, 2% glutaraldehyde in Hanks balanced salt solution, pH 7.3, was added and the pellet was broken into large clumps. Tubes were incubated at 37°C for 15 min, followed by cooling to RT for 10 min prior to treatment with 1.0% osmium tetroxide–0.5% potassium ferrocyanide for 90 min at 4°C. Clumps of cells were dehydrated through graded ethanol and embedded in Polybed 812 resin (Polysciences). Ultrathin sections were cut and stained with uranyl acetate–lead citrate and observed in a Philips 301 electron microscope.

Phosphoryl β -galactosidase activity. Access to phosphoryl β -galactosidase activity on the cytoplasmic face of the plasma membrane was used as a marker of the integrity of the protoplast. To induce synthesis of the enzyme, strain 2017-78 was grown in FMC (33), with lactose substituted for glucose. Preliminary experiments showed that this strain would ferment lactose by induction of phosphoryl β -galactosidase far more effectively than strain 133-79 and remain platelet interactive. After harvesting and washing, cells were suspended to an optical density at 620 nm of 1.0 (2×10^9 cells per ml) and treated with 40 U of mutanolysin per ml. Protoplasts were cultured in stabilized FMC for 6 days. Aliquots of protoplast culture (days 0, 2, 4, and 6) were removed and assayed for phosphoryl β -galactosidase activ-

ity by using the synthetic substrate *o*-nitrophenyl- β -D-galactopyranoside-6-phosphate (ONPG-6P) (Sigma) as modified from the method of Thompson (34). Protoplasts (4.8 ml) were brought to 30°C, and 200 μl of 0.1 M ONPG-6P was added and incubated for 20 min. Hydrolysis of substrate was stopped, and color was enhanced by the addition of 1.0 ml of 1.0 M Na_2CO_3 . Following centrifugation ($12,000 \times g$, 10 min), supernatant A_{420} was read by using a Beckman model 24 spectrophotometer. Hydrolysis of ONPG-6P was estimated from an ONP standard curve. Aliquots of protoplasts and control suspensions of bacterial cells were also permeabilized by duplicate freeze-thaw cycles, followed by treatment with 10 μl of toluene-acetone (1:4, vol/vol) per ml with vigorous vortexing for 2 min. These samples were then assayed for enzyme activity and compared with nonpermeabilized samples.

Platelet aggregometry. *S. sanguis* was tested for the ability to induce platelet aggregation with fresh platelet-rich plasma (PRP) obtained from a single donor by methods described in previous reports (7, 15, 16). In some experiments, 0.4 ml of PRP was preincubated for 10 min at 37°C in stirred cuvettes with 0.05 ml of soluble materials exported from protoplasts into FMC or cleaved from whole cells by trypsinization. In all cases, bacterial suspension (0.05 ml containing 2×10^8 cells) was added to the respective PRP suspensions. The lag time to onset of a wave of PRP aggregation was measured at 37°C in a recording aggregometer (model 660; Chronolog Corp., Havertown, Pa.). Percent inhibition was computed by subtracting control lag times from experimental lag times, dividing by the control, and multiplying that ratio by 100.

Platelet bacterial adhesion assay. The platelet bacterial adhesion assay was performed as previously described (15, 16), by using platelet ghosts that were prepared from Na_2EDTA -washed outdated human platelets (courtesy of Robert Bowman, American Red Cross Blood Center, St. Paul, Minn.). Bacterial cells, harvested and washed as described above, were suspended to a concentration of $4 \times 10^9/ml$ in 0.05 M Tris hydrochloride, pH 7.25, with 0.1 M $NaCl$ –1% Na_2EDTA –0.01 M NaN_3 . Platelet ghosts and washed bacteria were incubated together or alone in $2 \times$ volumes (controls) in V wells of microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.) for 30 min at 4°C. The clusters of adhering platelet ghosts and *S. sanguis* were sedimented by centrifugation ($55 \times g$, 20 min, 4°C), and adherence was determined by spectrophotometric analysis of the supernatant at 620 nm. In some cases, platelet ghosts were preincubated with soluble bacterial or protoplast components for 10 min at 37°C prior to incubation in microtiter plates. Percent inhibition was computed as 100 times the difference from control percent adhesion divided by the control percent adhesion.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as previously described (17) and as modified for vertical slab gels (9a). Samples were prepared in 1% (wt/vol) SDS sample buffer without 2-mercaptoethanol, incubated for 2 min in a boiling water bath, and electrophoresed on gradient gels of 5 to 10% acrylamide. Gels were then washed, fixed overnight in methanol, and stained with metachromatic silver (24).

Immunodiffusion. The cross-reactivity of class II antigen preparations was tested by immunodiffusion in a reaction with antiserum to *S. sanguis* I 133-79 cells. The antiserum was prepared and analyzed as in previous studies (9, 15–18).

Isolation of class II antigen from protoplast culture media by using immunoaffinity chromatography. After testing crude fractions, the supernatant of spent protoplast culture me-

dium (PpSup) was identified as a potential source of activity inhibitory to *S. sanguis*-induced platelet aggregation. After dialysis and lyophilization, released materials from 100 ml of protoplasts cultured in osmotically stabilized FMC were reconstituted to 5 mg of salt-free dry weight per ml in 0.1 M Tris buffer, pH 8.3. Class II antigen was purified by chromatography on an anti-class II immunoaffinity column. The preparation and properties of this affinity-purified rabbit polyclonal reagent and immunoaffinity matrix have been described elsewhere (9, 9a). The Tris buffer fall-through and 2 N NaSCN eluting samples were collected, dialyzed, lyophilized, and tested for interactions with platelets.

Isolation of class II antigen from protoplast culture media by using gel filtration and ion-exchange chromatography. *S. sanguis* PpSup was collected daily from 500-ml cultures as described above. These PpSup products were concentrated by ultrafiltration (XM50 membrane, 50,000 MWCO; Amicon Corp.). Next the concentrate was fractionated by a 45%–65% saturation of ammonium sulfate. The precipitate was then dissolved in dH₂O and dialyzed against 20 volumes of 0.2% Na₂EDTA, followed by 40 volumes of dH₂O, and lyophilized. The recovered solids were dissolved in 50 mM Tris, pH 8.0, with 20 mM NaCl. The sample was placed on a column (1.75 by 93 cm) of Sephadex G100 and chromatographed into 3-ml fractions at a flow rate of 16 ml/h. Fractions were analyzed for A₂₈₀, pooled, and assayed for platelet interactions by aggregometry after preincubation with PRP.

The fraction with the greatest ability to inhibit *S. sanguis*-induced PRP aggregation (G100-III) was concentrated by ultrafiltration (PM10 membrane) and further fractionated on a column (1 by 15 cm) of DEAE-Sephadex by using the chromatography protocol described elsewhere (9a). Fractions were pooled on the basis of A₂₈₀, concentrated (PM10 membrane), desalted, and tested for interactions with platelets.

TPCK-trypsinization of the protoplast class II antigens. Materials eluting with 2 N NaSCN from the anti-class II column or isolated by gel filtration and ion-exchange chromatography were reconstituted to 5 mg/ml in 0.2 M NH₄HCO₃ with 2.5 U of insoluble tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (Sigma) per ml. The sample was incubated by gentle tumbling for 10 min at 37°C. The insoluble trypsin beads were removed by centrifugation at 500 × *g* for 5 min. The resulting supernatant was dialyzed, lyophilized, and tested for interactions with platelets. Digestion was confirmed by SDS-PAGE.

Compositional analyses. Amino acids were analyzed by following the high-pressure liquid chromatography procedures of Bidlingmeyer et al. (3), as described elsewhere (9a). Neutral sugars (2) and hexosamines (32) were analyzed as their dansyl hydrazone derivatives, as described elsewhere (9a). Glycerol was determined by colorimetric assay involving coupled enzymatic reactions catalyzed by glycerolkinase, glycerolphosphate oxidase, and peroxide (Sigma Diagnostics, bulletin no. 337, 1989) after acid hydrolysis in 2 M HCl at 120°C for 48 h (19). Phosphorus was determined after digestion with 70% perchloric acid by using the ascorbic acid method of Chen et al. (6). Phosphomonoesters were determined after release by sulfuric acid hydrolysis (5) or cleavage with alkaline phosphatase (23), protein precipitation by trichloroacetic acid, and phosphorus analysis. Phosphodiester were determined similarly after hydrofluoric acid treatment (5) or conversion to phosphomonoesters and cleavage with alkaline phosphatase (23). Total lipids were estimated by the difference in weights before and after

extraction with chloroform and methanol (2:1, vol/vol). The presence of nucleic acids was estimated by A₂₆₀.

N-terminal analysis. Amino-terminal sequence analyses were performed by automated Edman degradation, by using an Applied Biosystems model 470A protein sequencer with on-line, reverse-phase chromatographic analysis of the phenylthiohydantoin derivatives as described elsewhere (9a).

Identification of blocked N-terminal amino acids. Initial automated N-terminal sequence analysis suggested that the amino terminus of the 115-kDa class II antigen may be blocked. Since nascent proteins may exist in PpSup, the presence of *N*-formylmethionine (fMet) was investigated. The 115-kDa class II antigen was treated with leucine aminopeptidase (LAP; 1:50, enzyme/substrate weight ratio) in 0.1 M Tris hydrochloride, pH 8.5, with 0.2 M MgCl₂ for 4 h at 40°C to release several amino acids at the N terminus (29). The released amino acids were separated from peptide with a Centricon C-10 filtration system (Amicon) and lyophilized. The sample was then derivatized and analyzed for phenylisothiocyanate (PITC)-amino acids as described elsewhere (9a). Amino acid standards, fMet and hydrolyzed fMet-Leu-Phe were used as controls. The 115-kDa class II antigen was then deformylated under vacuum with 0.5 M HCl in methanol (24 h, RT) and further analyzed for the presence of PITC-amino acids. Confirmation of the presence of fMet was established by thin-layer chromatography. Samples (200 µg) of the 115-kDa class II antigen and deformylated 115-kDa antigen were treated with LAP as described above, and the released amino acids were isolated, lyophilized, dissolved in 0.1 M HCl, and spotted (2 µl) onto cellulose sheets (Cellulose PEI 20 × 20 [0.1 mM thick]; Baker Scientific, Phillipsburg, N.J.). The amino acids were then separated with a solvent solution of isopropanol-formic acid-water (20:1:5, vol/vol/vol), as described by Niederwieser et al. (25).

RESULTS

Protoplasts of *S. sanguis* I 133-79. When surveyed by transmission electron microscopy, initial protoplast preparations showed numerous protoplasts and cell wall fragments among rare intact cells. The ultrastructure of untreated and mutanolysin-digested cells of *S. sanguis* I 133-79 were compared (Fig. 1). The bilaminar 9-nm cell membrane (Fig. 1, arrows and insert) within the wall of an intact cell (Fig. 1A) circumscribed a representative swollen, intact protoplast (Fig. 1B). Protoplasts with normal-appearing cytoplasm (Fig. 1B) were seen at the periphery of the field. Typical protoplasts were devoid of cell walls and associated fibrillar structures (Fig. 1C, higher magnification). Up to 99.4% of osmotically stabilized mutanolysin-digested cells of *S. sanguis* were estimated to possess this protoplast morphology.

The integrity of the plasma membranes of protoplasts introduced to our culture systems was assessed by measuring the access of a synthetic substrate to the inducible enzyme phosphoryl β-galactosidase. For these experiments, strain 2017-78 was substituted for 133-79. In pilot experiments, we learned that the replacement strain metabolized lactose more efficiently. Both strains are biotype I (11) and show similar platelet-interactive phenotypes (31). After growth and harvest from lactose-FMC, permeabilized and nonpermeabilized untreated cell and protoplast suspensions were compared for their abilities to hydrolyze ONPG-6P (Table 1). By comparing the micromoles of ONPG-6P hydrolyzed per cell per 20 min, 7% of untreated cells and 34%

FIG. 1. Transmission electron microscopy of untreated (A) and mutanolysin-digested (B and C) *S. sanguis* I 133-79. Plasma membrane is indicated by the arrows. Treated cells were digested for 75 min with 80 U/ml, as described in Materials and Methods. Arrows in the panel A insert show 9-nm cell membrane in a higher magnification of the untreated cell wall. Arrows in panels B and C (higher magnification) indicate the 9-nm-thick bilaminar membrane after digestion with mutanolysin. Bars, 0.1 μ m.

of the newly prepared day 0 protoplasts were estimated to be leaky. By day 4 of culture in lactose-FMC, 92% of protoplasts were leaky, reaching 100% by day 6.

Isolation of the class II antigen from *S. sanguis* protoplasts. Each day protoplasts in FMC were gently sedimented, spent medium (PpSup) was saved and analyzed for total protein, and cultures were reestablished with fresh medium. PpSup from days 1 to 4 contained copious amounts of protein (Fig. 2). By days 6 and 7, the protoplasts were difficult to

resuspend uniformly and the levels of soluble protein declined to zero. PpSup recovered on days 1 to 5 also inhibited the aggregation of platelets by *S. sanguis* cells. Uninoculated FMC was without effect. These protoplast-derived materials did not appear to affect *S. sanguis*-platelet adhesion (data not shown). The weight ratio of class II antigenic materials to total protein remained 0.05 through day 2, declining to 0.0125 on day 5, suggesting that the release of proteins into the medium was somewhat selective (Fig. 2).

TABLE 1. Integrity of *S. sanguis* protoplasts: hydrolysis of ONPG-6P by the cytoplasmic enzyme phosphoryl β -galactosidase^a

<i>S. sanguis</i> prepn	μmol (10^{-12}) of ONPG-6P hydrolyzed/cell per 20 min	% Leaky
Untreated cells		7
Nonpermeabilized	8.3	
Permeabilized	122	
Protoplasts (day 0)		34
Nonpermeabilized	39	
Permeabilized	116	
Protoplasts (day 2)		
Nonpermeabilized	35	
Permeabilized		
Protoplasts (day 4)		92
Nonpermeabilized	35	
Permeabilized	38	
Protoplasts (day 6)		100
Nonpermeabilized	21	
Permeabilized	19	

^a *S. sanguis* I 2017-78 was grown in lactose-containing medium, and protoplasts were prepared. On the days indicated, aliquots of each preparation were permeabilized in toluene-acetone. After incubation with ONPG-6P, the ratio of the rates of hydrolysis for nonpermeabilized and permeabilized forms times 100 indicated the percent leaky. Details are provided in Materials and Methods.

Pooled PpSup was chromatographed on an anti-class II immunoaffinity column. Of the applied protein, 5.2% bound to the matrix and was eluted with 2 N sodium thiocyanate (Table 2). When PRP was pretreated with a final concentration of 10 μg of the SCN⁻ eluate per ml, aggregation in response to *S. sanguis* cells was completely abrogated (Fig. 3). Unfractionated PpSup contained less class II antigen-like activity per microgram of protein, and virtually no activity was detected in the immunoaffinity column fall-through fraction. None of the anti-class II fractions affected platelet adhesion to cells of *S. sanguis*.

To isolate larger quantities of class II antigenic materials, PpSup was chromatographed on a column of Sephadex G100, pooled (Fig. 4A), and analyzed for platelet interactive activity. Fraction III contained the greatest amount of activity, inhibiting *S. sanguis*-induced platelet aggregation by

FIG. 2. Release of total protein and class II antigen(s) by *S. sanguis* protoplasts. Every 24 h, the protoplasts were sedimented ($15,000 \times g$, 30 min, RT) and resuspended in fresh medium (25 ml). The PpSup was saved, dialyzed, lyophilized, and assayed for total soluble protein. The protoplast products collected each day were placed on the anti-class II immunoaffinity column. The retained, SCN⁻ eluting materials were collected, dialyzed, lyophilized, and assayed for protein.

TABLE 2. Isolation of the class II antigen by immunoaffinity chromatography^a

Sample	Protein (mg)	% Recovery
Protoplast spent medium	5.00	
Tris buffer fall-through	4.63	92.6
SCN ⁻ eluate	0.26	5.2
Total		97.8

^a An anti-class II immunoaffinity column was prepared by coupling 15 mg of gamma globulin from rabbit anti-class II to 20 ml of cyanogen bromide-activated Sepharose 4B (0.8 by 20 cm). Spent PpSup protein (5 mg) was dissolved in 1 ml of 0.1 M Tris, pH 8.3. The Tris buffer fall-through and 2 N NaSCN eluting samples were collected in batches (200 ml each).

78.3%. Sephadex G100-III was then chromatographed on a column of DEAE-Sephadex, which was eluted with a linear gradient of 20 to 750 mM of NaCl in 50 mM Tris, pH 8.0, as described previously (9a). Fraction 7 from DEAE-Sephadex completely inhibited platelet aggregation induced by intact *S. sanguis* cells at a concentration of 5 $\mu\text{g}/\text{ml}$. After DEAE-Sephadex rechromatography (Table 3), the purified class II material, with a 50% inhibitory dose of 1.7 $\mu\text{g}/\text{ml}$, was 30 times more inhibitory to platelet aggregation than the PpSup.

Comparison of class II antigens isolated from PpSup and the surface of cells of *S. sanguis*. Fractions of PpSup products and the 65-kDa class II antigen of cells of *S. sanguis* isolated by immunoaffinity chromatography from minimal TPCK-trypsin digests were compared by SDS-PAGE (Fig. 5). The cell-derived 65-kDa class II antigen (0.5 μg ; Fig. 5, lane A) was not apparent in the PpSup after dialysis (lane B) or the PpSup fall-through fraction of the anti-class II immunoaffinity column (lane C). Note that a band with similar, but nonidentical, R_f (lanes B and C) was unaffected by immunoaffinity chromatography and had different metachromatic staining properties than authentic class II antigen. After TPCK-trypsinization, 0.5 μg of the SCN⁻ eluting fraction

FIG. 3. Effect of soluble protoplast products on platelet aggregation in response to *S. sanguis*. After dialysis and lyophilization, soluble protoplast products were reconstituted in 0.1 M Tris buffer, pH 8.3, and placed on the anti-class II immunoaffinity column. The fall-through and 2 N NaSCN eluting samples were collected, dialyzed, lyophilized, and tested for the ability to inhibit *S. sanguis*-induced platelet aggregation.

FIG. 4. (A) Gel filtration chromatography of spent media from protoplast cultures of *S. sanguis* I 133-79. Released protein (33 mg) was placed on a column of Sephadex G100 and chromatographed as described in Materials and Methods. (B) Ion-exchange chromatography of G100 fraction III. After dialysis and lyophilization, 17.1 mg of protein was placed on a column of DEAE-Sephadex and chromatographed as described in Materials and Methods.

showed a single 65-kDa band (lane D). Both 65-kDa class II antigen preparations (Fig. 6, wells a and b) showed a reaction of identity with affinity-purified rabbit anti-class II serum (unlabeled well) in immunodiffusion. Prior to trypsinization of the SCN^- eluting fraction, at least one additional cross-reactive, faintly stained antigen was present (well c, arrowhead).

The 65-kDa protein fragment previously purified from intact cells of *S. sanguis* (9a), class II PpSup materials eluted from the anti-class II matrix by SCN^- , and fraction 7 from DEAE from the sequential column chromatography protocol each showed nearly identical dose-response inhibition of platelet aggregation. At a final concentration of 1 $\mu\text{g}/\text{ml}$, the three preparations inhibited between 7.9 and 10.1%. Minimal trypsinization of materials from PpSup did not affect their inhibitory activity, and no differences in their chemical compositions were apparent (Table 4).

Isolation of platelet-interactive 115-kDa class II protein from PpSup by sequential column chromatography was analyzed by 5 to 10% gradient SDS-PAGE after silver staining (Fig. 7). Numerous bands were present in the PpSup (Fig. 7, lane A). After ultrafiltration and ammonium sulfate fractionation, more than 14 bands were resolved (lane B). Fraction III from Sephadex G100 showed components in the range of 50 to 150 kDa (lane C). After ion-exchange chro-

matography, DEAE fraction 7 showed a single band of 115 kDa (lane D). After TPCK-trypsinization, this fraction showed a major 65-kDa band plus several other minor bands (lane E).

The 115-kDa material showed proportionally less protein and more carbohydrate than the cell-derived 65-kDa protein (Table 4). Amino acid analysis showed that the compositions of both forms were similar and that Glu, Gly, and Lys were the major amino acids. While the 65-kDa protein contained 4.0% carbohydrate, neither *N*-acetylglucosamine nor *N*-acetylgalactosamine, the most likely linkage sugars to peptide, was detectable. The 115-kDa protein did contain these

TABLE 3. Steps to purification of the class II antigen

Step ^a	Total protein, mg (%) ^b	ID ₅₀ ($\mu\text{g}/\text{ml}$) of platelet aggregation ^c
Released proteins	400	88
Ultrafiltration	203 (50.7)	46
45%-65% Ammonium sulfate	97.6 (24.4)	24
Sephadex G100	17.1 (4.3)	4.3
DEAE-Sephadex	5.7 (1.4)	2.3
DEAE rechromatography	4.2 (1.0)	1.7

^a Protoplast proteins released daily into spent medium were collected, concentrated by ultrafiltration (XM50, 50,000 MWCO), fractionated with 45%-65% saturated ammonium sulfate, and lyophilized. The resulting proteins were chromatographed on a column of Sephadex G100 as described above. The active fraction was then chromatographed on an ion-exchange column. A second cycle on this DEAE-Sephadex column was necessary for the degree of purification obtained.

^b Percent total applied protein recovered in each of the purification steps.

^c ID₅₀ (50% inhibitory dose) for platelet aggregation was estimated from dose-response inhibition experiments.

FIG. 5. Analysis of released protoplast products by SDS-PAGE (5 to 10% acrylamide). Lanes: A, 65-kDa class II antigen isolated by anti-class II immunoaffinity chromatography from tryptic digests of *S. sanguis* cells; B, released macromolecular fraction from PpSup; C, anti-class II immunoaffinity fall-through of PpSup; D, thiocyanate eluting fraction after TPCK trypsinization. Lanes A and D each contained 0.5 μg of protein; lanes B and C each contained 1 μg of total protein. All samples were solubilized in 1% (wt/vol) SDS sample buffer, electrophoresed, and stained with metachromatic silver.

FIG. 6. Demonstration of immunological cross-reactivity between class II antigens from *S. sanguis* cells and released protein from protoplasts. By using Ouchterlony immunodiffusion, class II antigen prepared from *S. sanguis* I 133-79 cells by 3 min of TPCK-trypsin digestion (a; 10 µg of protein), class II antigen from protoplasts after trypsinization (b; 10 µg of protein), and protoplast class II antigen before trypsinization (c; 10 µg of protein) were reacted with affinity-purified rabbit anti-class II (unlabeled well, 50 µg of protein). Precipitins were stained with Coomassie blue. The arrowhead indicates the position of faintly stained additional precipitin.

hexosamines, and almost half of the residue percent dry weight of carbohydrate was rhamnose. While no phosphorus was detected in the 65-kDa protein, the 115-kDa protein contained 1.2% phosphorus present in phosphodiester linkages.

N-terminal analysis of the 115-kDa class II antigen. The 115-kDa antigen was digested with LAP to release amino acids that appeared blocked upon automated N-terminal sequence analysis. The residual protein showed a single band on SDS-PAGE (data not shown) and no change in inhibitory activity in platelet aggregation. Among the 15 to 20 residues released by LAP, the presence of formyl methionine was verified by comparison to PITC-amino acid analysis of standard fMet. The first (13.33 min) of dual peaks corresponded to methionine. The second peak (15.35 min) was assumed to represent the retention time for fMet. One residue released from LAP-treated 115-kDa class II protein had the retention time of fMet. Removal of the formyl group prior to amino acid analysis resulted in a decreased amount of detectable fMet and an increase in methionine. The presence of fMet among the amino-terminal residues of the 115-kDa class II antigen released by LAP was confirmed by thin-layer chromatography, which showed a distinct fMet spot. After deformylation in alcohol, the sample contained no fMet and an increase in Met was noted.

DISCUSSION

We have been studying the mechanism of platelet aggregation induced in vitro by interactions with the surface of *S. sanguis*. Since the action of whole *S. sanguis* cells can be mimicked by their washed cell walls (15, 18), we have focused our attention on the identification of platelet-interactive surface determinants. Ultrastructural studies have shown that electron-dense fibrils, cleavable from the cell wall by trypsin, probably interact with the platelet plasma membrane (16). Morphologically, and perhaps structurally, these fibrils are similar to those identified on other strains of *S. sanguis* (10) and related organisms such as *S. faecalis* (14) and M-protein-positive group A streptococci (27). However, after release from the cell wall by trypsinization, *S. sanguis* protein fragments were unable to directly induce any measurable platelet response. They did, however, react with platelets to inhibit responses induced by whole cells or cell walls of *S. sanguis* (16).

TABLE 4. Chemical composition of 65- and 115-kDa forms of the class II antigens

Constituent(s)	Antigen			
	65 kDa ^a		115 kDa	
	% Dry wt	Estimated residues/molecule	% Dry wt	Estimated residues/molecule
Amino acids				
Alanine	5.0	45	3.7	60
Arginine	6.6	27	3.5	27
Aspartic acid	5.5	31	4.0	40
Cysteine	1.0	4	0.7	5
Glutamic acid	11.1	55	8.3	74
Glycine	14.7	166	8.2	166
Histidine	1.8	8	1.1	9
Isoleucine	2.7	16	1.8	18
Leucine	3.2	18	2.8	28
Lysine	13.6	68	8.4	75
Methionine	0.6	3	1.0	9
Phenylalanine	3.5	15	2.0	16
Proline	7.5	50	4.4	52
Serine	3.0	22	2.3	30
Threonine	2.6	17	2.0	23
Tyrosine	1.7	7	1.8	13
Valine	5.2	34	3.1	36
Subtotal	89.2	586	59.1	681
Carbohydrate				
Galactose	1.2	4	7.9	50
Glucose	1.7	6	3.5	22
Mannose	0.3	1	5.1	32
Ribose	0.2	1	1.3	10
Rhamnose	0.6	2	18.2	128
N-acetylglucosamine	0	0	1.9	9
N-acetylgalactosamine	0	0	1.1	6
Muramic acid			<0.02 ^b	
Subtotal	4.0	14	39.0	257
Glycerol			0.5	5.8
Nucleic acids	0		0	
Lipids	<2 ^b		<2 ^b	
Phosphorus	0		1.2	44
Phosphomonoesters	0		0.01	1
Phosphodiester	0		1.03	38
Total	93.2		99.8	

^a Cell-derived 65-kDa antigen (9a).

^b Below the limit of detection.

Affinity-purified anti-*S. sanguis* antibodies reacted with whole cells to block interactions with platelets. The same antibodies also neutralized the platelet-inhibitory activity of the released fragments. Hence, the cell-free protein fragments were derived from the platelet-interactive antigenic determinants on the cell wall. These cell wall antigens include a collagenlike determinant that is immunologically cross-reactive with the platelet-interactive domains on types I and III collagen (9). Not only do anti-*S. sanguis* antibodies inhibit collagen-induced platelet aggregation (9), but human platelets that are specifically defective in responding to collagen also fail to respond to cells of *S. sanguis* (31).

To obtain more native forms of the collagenlike class II antigen(s) for study, we hypothesized that the medium of cultured, cell wall deficient protoplasts of *S. sanguis* would be a good source. In this paper, we show that mutanolysin effectively digested the peptidoglycan of *S. sanguis* such that osmotically sensitive protoplasts were prepared. In our

FIG. 7. Analysis of the isolation of 115-kDa class II antigen by 5 to 10% gradient SDS-PAGE stained with silver. Each lane contained 1 μ g of the following: spent protoplast culture medium (A), proteins after ultrafiltration and fractionation with ammonium sulfate (B), Sephadex G100 fraction III (C), and rechromatographed DEAE fraction 7 before (D) and after (E) TPCK-trypsinization. All samples were solubilized in 1% (wt/vol) SDS sample buffer, electrophoresed, and stained with metachromatic silver as described in Materials and Methods.

hands, other muralytic enzymes, such as lysozyme and lysostaphin, even when used together, could not produce osmotically sensitive *S. sanguis* (data not shown). During protoplast formation, cells were stabilized osmotically in 26% raffinose (36). During formation, protoplasts were recovered in a mixture with large fragments of cell wall. Protoplasts at the time of preparation had a 9-nm-thick plasma membrane. For culture, the protoplasts were stabilized in chemically-defined synthetic FMC with 0.5 M ammonium chloride. Penicillin was added to prevent cell wall reassembly (26, 35). Since FMC was completely dialyzable, the proteins and other macromolecular products produced by the continuously cultured protoplasts could be conveniently isolated. During the 6- to 7-day period of isosmotic culture, protoplasts became increasingly permeable to ONPG-6P. Hence, it is still unclear that the proteins released into the PpSup after each day of culture were actually exported. The maximal release of proteins into the PpSup occurred while the protoplasts were largely intact, suggesting that during the first few days export may have occurred.

At least four macromolecular products of greater than 65,000 daltons, the molecular mass of the purified class II antigen obtained by minimal trypsinization of the whole cell (9, 9a), were then isolated from PpSup by reaction with an anti-class II immunoaffinity matrix. Only one of these, a 115-kDa class II antigen, was isolated by sequential column chromatography. By using TPCK-trypsin, these components hydrolyzed to yield a single 65-kDa protein. This protein was immunologically identical to the platelet-interactive antigen we purified earlier from minimal tryptic digests of *S. sanguis* cells (9a). By immunodiffusion, at least one other cross-

reactive antigen from protoplasts was detected prior to trypsinization of the class II antigen preparation.

The 115-kDa form has amino-terminal fMet, indicating the presence of signal peptide. Since this peptide would be cleaved before export, this protein is probably released from leaky protoplasts into the medium. The 115-kDa class II antigen is also associated with a high percentage by weight of carbohydrate, is rich in rhamnose, and shows a single band of 115 kDa on periodic acid-Schiff-stained SDS-PAGE (data not shown). A rhamnose-rich surface glycoprotein would be highly unusual for a bacterial cell. In contrast, rhamnose polymers are often covalently associated with peptidoglycans or cell surface amphiphiles.

The structure of rhamnose residues and their association with the 115-kDa class II antigen remains to be clarified. The carbohydrate remained associated with protein after ion-exchange chromatography and SDS-PAGE. The chemical composition of more than 98% of the residue dry weight was amino acids and carbohydrate (Table 4). While 44 residues per molecule were phosphorus (38 residues in phosphodiester linkage), only 5.8 residues of glycerol per molecule were released by acid hydrolysis. Lipid and muramic acid, if present, were below the limits of detection. This 115-kDa *S. sanguis* antigen appeared essentially free of peptidoglycan and lipoteichoic acid. Thirty-fold more LTA-like amphiphiles, marked by [3 H]glycerol in the aqueous phase of phenol extractions, were recovered from nonplatelet-interactive strain M5 than from strain 133-79 (M. C. Herzberg and K. L. Brintzenhofe, J. Dent. Res. 62(Spec. issue):abstr. 199, 1983). Aqueous phenol preparations from strain 133-79 also failed to react with anti-polyglycerolphosphate antisera (gifts of Ken Knox, Institute of Dental Research, United Dental Hospital of Sydney, New South Wales, Australia, and Burton Rosan, School of Dental Medicine, University of Pennsylvania, Philadelphia) and had 1/20 of the specific platelet-inhibitory activity recovered in crude minimal tryptic digests. It is likely therefore that the 115-kDa antigen is associated with rhamnose-rich polysaccharide units linked together by phosphodiester bonds, characteristic of many streptococci (1). The platelet-inhibitory activity has been shown to reside in a proline-rich, collagenlike 23-kDa peptide cleaved from the 65-kDa fragment released from whole cells by trypsin (9a). The abundant carbohydrates found associated with the 115-kDa protein do not apparently contribute to this function. To understand how these surface proteins are configured on the cell wall to induce platelet aggregation, further analysis of the structural properties of the larger macromolecule is warranted.

The electron-dense surface fibrils of platelet-interactive streptococci appear to carry the Agg⁺ phenotype (16). While the platelet-interactive protein may be the product of one gene, the fibrils are the products of many genes. The association of these gene products into fibrils may be modelled in part by the 115-kDa material with its associated carbohydrate. The protoplast culture system affords a unique opportunity to isolate this material for analysis.

About 5% of the total released macromolecular material reacts with anti-class II antigen, and about 1% was isolated as the 115-kDa form of the class II antigen. From 2 liters of protoplast culture, about 4 mg of class II antigenic protein can be isolated each week. Hence, the protoplast culture technique will facilitate future studies of the structure and function of *S. sanguis* platelet-interactive proteins.

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