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Isolation of a Heart- and Kidney-Binding Protein from Group A Streptococci

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Tritium-labeled, water-soluble components of *Streptococcus pyogenes* type M6 adsorbed to cardiac tissue in vitro. Tissue binding was time dependent, saturable, and reversible. Chromatography of the crude bacterial extract on Bio-Gel P-300 indicated a molecular weight greater than 300,000 for the heart-binding component. Sodium dodecyl sulfate (SDS) dissociated this aggregate into a protein of 18,000 to 20,000 daltons as determined by Sephacryl S-200 chromatography and SDS-polyacrylamide disc gel electrophoresis. The tissue-binding protein was also purified from streptococcal extracts by adsorption to immobilized heart components. SDS-polyacrylamide gel electrophoresis of the protein desorbed from tissue revealed a radioactive band of 19,000 daltons. Indirect immunofluorescence tests on cardiac tissue treated with streptococcal extract showed an accumulation of a bacterial antigen on the sarcolemmal sheaths. Streptococcal components also adsorbed to basement membranes of kidney. Antisera prepared to isolated cytoplasmic membranes and water-soluble extracts of *S. pyogenes* type M6 were the most sensitive reagents for the detection of bacterial components bound to tissue. Antisera prepared to isolated cell walls and to intact bacteria were weakly reactive in these assays.

The extracellular products and surface components of group A streptococci have been studied in recent years in an attempt to define the virulence factors responsible for rheumatic fever and poststreptococcal glomerulonephritis. A variety of substances have been found to induce heart or kidney pathology when injected into laboratory animals, including streptolysins S and O, the erythrogenic toxins, protease (17, 18, 31), and lipoteichoic acid (13, 17, 30). A cell-bound antigen (19) and an extracellular protein (29) have been detected that are unique to nephritogenic strains of group A streptococci. Although these latter substances appear in the glomeruli of patients with acute poststreptococcal glomerulonephritis, their roles in the pathogenesis of the disease have not been defined.

We recently reported (28) on a protease-sensitive surface component (s) of *Streptococcus pyogenes* type M6 that bound to cardiac and skeletal muscle in vitro. Indirect immunofluorescence (IIF) staining indicated that the bacterial antigen selectively adsorbed to components of the sarcolemmal sheaths. This binding property indicates that this streptococcal component could act as a virulence factor in rheumatic fever. In this communication, we report the in vitro binding of a streptococcal protein antigen to kidney basement membranes, as detected by IIF, and describe direct binding of this streptococcal protein to heart muscle. Some characteristics of the partially purified tissue-binding protein are also reported.

MATERIALS AND METHODS

Bacteria and cultural conditions. *S. pyogenes* type M6 (ATCC 12348), *S. pyogenes* type M12 (ATCC 12351), *S. sanguis* (ATCC 10556), and *Staphylococcus aureus* (ATCC 12600) were grown in tryptic soy broth supplemented with 0.1% yeast extract (Difco Laboratories, Detroit, Mich.).

All cultures (1 liter of medium in a 2-liter Erlenmeyer flask) were incubated at 37°C and mixed at 25 rpm on a gyratory shaker (New Brunswick Scientific Co., New Brunswick, N.J.). Cell growth was followed turbidimetrically at 600 nm, using a Bausch & Lomb Spectronic 20 spectrophotometer. Cultures with optical densities of 0.8 to 1.2 (late logarithmic to early stationary phases of growth) were harvested by centrifugation at 10,000 × *g* for 15 min at 4°C. The cells were then washed twice with 0.01 M NaH_2PO_4-Na_2HPO_4, pH 7.2, containing 0.15 M NaCl (phosphate-buffered saline; PBS).

Fractionation of bacteria. Cells were suspended in PBS to a density of 100 mg (moist weight) per ml and then disrupted by shaking with glass beads in a Braun cell homogenizer (Bronwill Scientific Inc., Rochester, N.Y.) at 4°C as described by Bleiweis et al. (6). The lysed cell suspensions were centrifuged at 200 × *g* for 15 min to remove intact cells. Cell walls and cytoplasmic membranes were then removed by centrifugation at 105,000 × *g* for 1 h and purified as previously described (28). Ammonium sulfate was added to the

supernatant fluid, and the bacterial components that precipitated between 25 and 60% ammonium sulfate saturation were collected by centrifugation, dialyzed against distilled water, and lyophilized. Initial tissue-binding studies were conducted with this preparation reconstituted in PBS.

Subsequent studies established that the tissue-binding component could be extracted directly with alkali as described below. This procedure was routinely used since it was less time consuming, was more reproducible, and resulted in higher yields of the tissue-binding protein. Bacteria were suspended in acetone at a concentration of 100 mg (moist weight) per ml. After 30 min at room temperature, the residue was collected by centrifugation and reextracted with fresh acetone. The cell residue was then suspended in chloroform-methanol (2:1, vol/vol), stirred for 30 min at room temperature, and centrifuged. The resulting pellet was reextracted with fresh chloroform-methanol (2:1). The final pellet was then suspended in distilled water, and the pH was adjusted to and maintained at 10.0 with dilute ammonium hydroxide. The suspension was stirred for 18 h at room temperature. After centrifugation, the supernatant fluid was dialyzed against distilled water and lyophilized.

Immunization. Soluble cell extracts and suspensions of cell walls, cytoplasmic membranes, or killed whole cells (heated at 70°C for 30 min) were diluted in PBS to 10 mg (dry weight)/ml. White male New Zealand rabbits were injected intravenously with 0.1 ml of the appropriate vaccine three times per week for a period of 3 weeks. The animals were allowed to rest for 1 week, after which the immunization regimen was repeated. Rabbits with low serum titers were reimmunized for an additional 3 weeks.

Antibody purification. Goat anti-rabbit immunoglobulin G (IgG) immunoglobulins were isolated from whole serum by affinity chromatography, using rabbit immunoglobulin fraction II (Miles Research Products, Elkhart, Ind.) coupled to cyanogen bromide-activated Sepharose 4B beads (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) (28).

Immunofluorescence assay. Selected rabbit antisera were titrated by IIF on 4-μm-thick cryostat-cut sections of rhesus monkey (*Macaca mulatta*) tissue. Twofold serial dilutions of the antiserum in PBS (pH 7.4) containing 2% bovine serum albumin were incubated on the tissue sections for 30 min followed by a 15-min wash in PBS. The tissue was then incubated for 30 min with fluorescein isothiocyanate-labeled goat IgG specific for rabbit IgG followed by a PBS wash for 30 min. The goat anti-rabbit IgG conjugate had a molar fluorescein/protein ratio of 3.5 and 16 U of antibody/ml (5). In subsequent IIF assays, the labeled antibody preparation was used at 0.5 U of antibody/ml.

Rabbit antisera were also titered on tissue sections that were pretreated with bacterial cell extracts. For these assays, tissue sections were incubated with dilutions of cell extract for 30 min, washed with PBS for 15 min, and then stained by IIF as described above. The intensity of staining was graded on a scale of 0 to 4, with 4 signifying the greatest reaction.

Radioisotope labeling. Bacterial proteins were radiolabeled with [^{3}H]acetic anhydride (Amersham Corp., Arlington Heights, Ill.), using a modification of the procedure of Weber (33). Cell extracts, 150 mg (dry weight) in 30 ml of 0.1 M phosphate buffer, pH 8.5, were added to 15 mCi of [^{3}H]acetic anhydride (5.5 Ci/mmol) and stirred for 18 h at 4°C. The solution was dialyzed against distilled water to remove nonprotein reactants. The tritium-labeled preparation had a specific radioactivity of 4.6×10^6 cpm/mg of lyophilized dry weight.

Fractionation of heart tissue. Trimmed heart muscle (22 g) from six rhesus monkeys was cut into 5-mm chunks and blended in 100 ml of cold PBS (pH 7.4), using a Sorvall Omnimixer (DuPont Instruments, Nowalk, Conn.). The resulting suspension was filtered through multiple layers of gauze to remove connective tissue. The filtrate was then shaken with glass beads (0.1 to 0.11 mm) in a Braun cell homogenizer at 4°C. The homogenate was centrifuged at 105,000 × *g* for 1 h. The resulting pellet of cardiac tissue fragments was washed twice with PBS and stored at −20°C.

Binding assay. Tissue-binding activity of streptococcal cell components was determined by a radioisotope assay. A suspension of homogenized heart muscle (275 μg of protein in 0.2 ml) was dispensed into 7-ml glass scintillation vials, dried at 37°C for 24 h, and heat fixed at 56°C for 1 h. The tissue-coated vials were stored in a desiccator at room temperature for as long as 2 months without loss of activity. All other manipulations for the assay were conducted at room temperature.

The dried heart homogenate was incubated with 0.3 ml of PBS, pH 7.4, containing 1% bovine serum albumin. After 30 min the excess fluid was removed, and the moist heart homogenate was incubated for 60 min with 0.2 ml of ^{3}H-labeled bacterial extract (0.25 to 1 mg [dry weight]/ml). The vials were rinsed three times with 4 ml of PBS followed by a 30-min soaking wash in 4 ml of the buffer solution. The contents of the vials were solubilized with 0.5 ml of 10% Scintigest tissue solubilizer (Fisher Scientific Co., Rochester, N.Y.) at 56°C followed by the addition of 5 ml of liquid scintillation fluid (ScintiVerse; Fisher Scientific Co.). The radioactivity was determined in a Nuclear-Chicago Mark II liquid scintillation spectometer.

Gel filtration on Bio-Gel P300. Radiolabeled bacterial extract (10 mg) was reconstituted in 2 ml of PBS, pH 7.4, containing 0.05% sodium azide. After 2 h at 4°C the solution was clarified by centrifugation at 12,000 × *g* for 30 min and applied to a column (1.6 by 90 cm) of Bio-Gel P300 (Bio-Rad Laboratories, Richmond, Calif.) equilibrated with the same buffered solution. Fractions of 5 ml were collected at 4°C at a flow rate of 12 ml/h.

Gel filtration on Sephacryl S200. Radiolabeled bacterial components were dissolved at 10 mg/ml in 5% sodium dodecyl sulfate (SDS) and heated at 70°C for 1 h. The sample was clarified by centrifugation at 12,000 × *g* for 30 min and then applied to a column (1.6 by 60 cm) of Sephacryl S200 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) equilibrated with 0.05 M ammonium acetate, pH 7.2, containing 0.1% SDS. Fractions of 1 ml were collected at room temperature at a flow rate of 3 ml/h.

Electrophoresis. SDS-polyacrylamide gel electrophoresis was performed with 10% acrylamide gels, according to the method of Weber and Osborn (32). Lysozyme (14,300 daltons), lactoglobulin (18,400), chymotrypsinogen (25,000 daltons), pepsin (35,000 daltons), ovalbumin (45,000 daltons), and bovine serum albumin (67,000 daltons) were used as molecular weight standards (Sigma Chemical Co., St. Louis,

Mo.). Gels were stained with Coomassie brilliant blue or periodic acid-Schiff reagent as described by Segrest and Jackson (26).

Other assays. Total carbohydrate was determined by the phenol-sulfuric acid method of Dubois et al. (14). Protein was measured by the method of Lowry et al. (21), using bovine serum albumin as a standard. Dry weight was determined on preparations which were dialyzed against distilled water and lyophilized.

RESULTS

Kidney-binding activity. Previous studies had shown that certain extracts of *S. pyogenes* type M6 cells contained antigens that selectively bound to the sarcolemmal sheaths of skeletal and cardiac muscle fibers in vitro (28). A similar experiment was conducted in which isolated streptococcal components were incubated with cryostat-cut sections of rhesus monkey kidney. Subsequent IIF staining of the tissue with rabbit anti-*S. pyogenes* serum indicated that bacterial components were bound to the tubular and glomerular basement membranes (Fig. 1A). Tissue sections in the absence of added bacterial antigens did not react with the antistreptococcus serum (Fig. 1B), indicating that antibody binding in Fig. 1A was not the result of immunological cross-reactivity between *S. pyogenes* and tissue components. As an additional control, serum obtained before immunization (preimmune serum) was incubated with both untreated and streptococcal extract-treated tissue sections. No staining of basement membranes occurred upon subsequent IIF testing.

Rabbit antisera elicited by immunization with whole bacteria or selected cellular fractions were tested on untreated and streptococcal extract-treated kidney and cardiac muscle sections (Table 1). Anti-cell membrane and anti-whole cell extract (25 to 60% ammonium sulfate fraction) sera were the most sensitive reagents for the detection of the tissue-binding component of these group A streptococci.

Extracts of an M12 strain were also found to have kidney- and heart-binding activities. Tissue-bound antigen from the M12 strain was detected by IIF staining with anti-*S. pyogenes* M12 and anti-*S. pyogenes* M6 whole-cell sera. Similar preparations from *S. sanguis* and *Staphylococcus aureus* cells were nonreactive with homologous antisera and anti-*S. pyogenes* sera.

Direct binding assay. ^{3}H-labeled streptococcal components were tested for their ability to bind to cardiac muscle components (Fig. 2). Saturation of the tissue sites was observed at concentrations of ^{3}H-labeled bacterial extract above 4 mg/ml (0.8 mg/0.2 ml). Binding was also time dependent (Fig. 3).

The complex formed between streptococcal components and cardiac muscle components was not dissociated by washing the tissue fragments with PBS, pH 7.4, at room temperature for up to 24 h. The reversibility of the complex was also tested by using competitive dissociation (Fig. 4). After an initial period of binding using an incubation mixture containing 400 μg of ^{3}H-labeled bacterial components, varying amounts of nonradioactive bacterial components were added, and the mixtures were incubated for an additional 60 min. The quantity of tissue-bound radioactivity was decreased 65% by a 20-fold excess of competitor (13.4 mg/ml). Larger quantities of competitor did not cause a significant increase in dissociation of bound radioactivity.

The effect of pH and temperature alterations on the binding activity of the streptococcal component and the dissociation of the complex could not be fully determined because of variations in the solubilities of the reactants. Previous studies had shown the tissue-binding component *S. pyogenes* to be almost totally inactivated by heating at 56°C for 1 h (28). In subsequent studies, incubation at 37°C caused a gradual loss of activity over a period of 8 h. This inactivation appeared to be the result of insolubility rather than inactivation, because the effect was completely reversed by resolubilization of the precipitated material at pH 9.0. Binding activity of streptococcal extracts exhibited a broad pH optimum of 6 to 10. Below pH 6.0, precipitation occurred and binding activity was rapidly lost.

Purification of tissue-binding factor. Solubilized streptococcal components were radiolabeled with [^{3}H]acetic anhydride and subjected to gel filtration on Bio-Gel P300 (Fig. 5). Substances with heart- and kidney-binding activity were eluted near the void volume, indicating a molecular weight in excess of 300,000. This observation, together with the solubility changes in the extracted components during the pH and temperature experiments, indicated that the tissue-binding factor could form aggregates with other streptococcal components. In subsequent studies, we found that SDS was the most effective dissociating agent and that low detergent concentrations did not destroy the tissue-binding activity. Figure 6 shows separation of streptococcal components by Sephacryl S200 chromatography in the presence of 0.1% SDS. Tissue-binding activity was localized in peak B.

Examination of the peak B material by SDS-polyacrylamide disc gel electrophoresis (10% acrylamide) revealed multiple components (Fig. 7). The tissue-binding factor was identified by slicing an unstained gel and testing the eluted proteins for heart-binding activity. Molecular weights of 19,000 and 21,000 were estimated for the tissue-binding factor, using SDS-gel electrophoresis and SDS-Sephacryl S200 gel filtration, respectively.

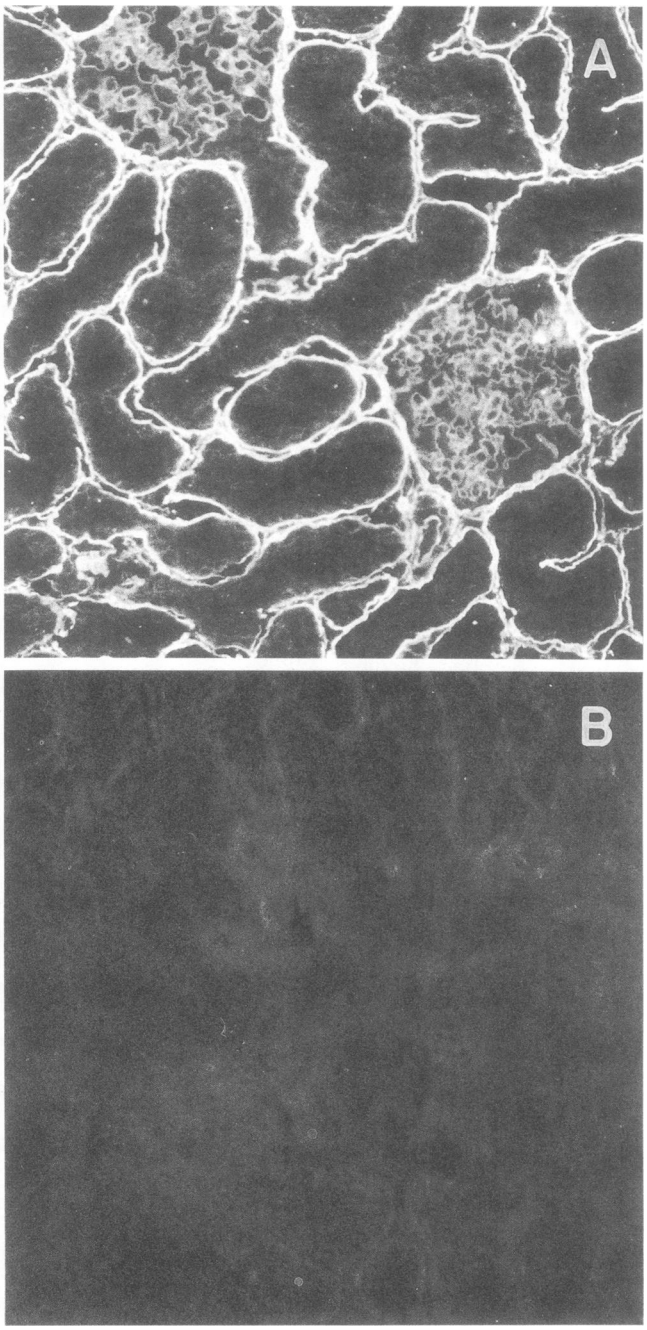

FIG. 1. Micrographs (×200) of monkey kidney stained by IIF. (A) Tissue section treated with solubilized streptococcal components (1 mg/ml) followed by rabbit anti-*S. pyogenes* cell extract serum (1:40). Tubular and glomerular basement membranes are stained. (B) Tissue section treated with a 1:40 dilution of rabbit anti-*S. pyogenes* cell extract serum.

The identity of the tissue-binding factor was verified by SDS-polyacrylamide disc gel electrophoresis after its adsorption to and desorption from heart tissue (Fig. 8). In this experiment, cardiac muscle fragments were incubated with ^{3}H-labeled streptococcal components (peak B material from Sephacryl S200 chromatography) and then washed with PBS to remove unbound radiolabeled substances. Bound bacterial components were solubilized by extraction of the

TABLE 1. IIF titration of rabbit sera on rhesus monkey kidney and cardiac muscle pretreated with solubilized *S. pyogenes* antigens

Serum	IIF titer[a]		
	Kidney		Heart sarcolemmal sheath
	GBM	TBM	
Anti-whole cell	4	16	80
Anti-cell wall	≤4	≤4	40
Anti-cell membrane	20	80	320
Anti-cell extract[b]	40	320	320
Normal rabbit sera	≤4	≤4	≤4

[a] Cryostat-cut tissue sections were pretreated with solubilized *S. pyogenes* M6 antigens (2 mg [dry weight]/ml) and then incubated with twofold serial dilutions of the indicated rabbit antisera. All sera were nonreactive at 1:4 dilutions on untreated (native) tissue. GBM, Glomerular basement membrane; TBM, tubular basement membrane.

[b] "Cell extract" refers to the antigens obtained by $(NH_4)_2SO_4$ precipitation (25 to 60% fraction) of whole-cell extracts.

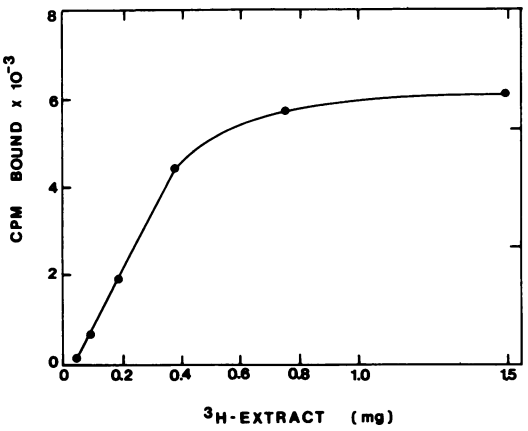

FIG. 2. Binding of ^3H-labeled streptococcal components to cardiac muscle fragments as a function of concentration. Binding was allowed to proceed at room temperature for 60 min. The indicated amounts are for 0.2-ml reaction mixtures.

complex with 1% SDS at 60°C for 1 h. A major band of radioactivity, with an apparent molecular weight of 19,000, was detected in the polyacrylamide gel after electrophoresis of the SDS-solubilized material. The radioactivity pattern of gel slices obtained after electrophoresis of peak B material is included for comparison. The electrophoretic mobility of the tissue-binding factor eluded from heart components (R_f 0.69) corresponded to that of the active component identified in Fig. 7.

DISCUSSION

The binding of streptococcal protein to tissue components can be accurately determined with the radioactive assay described in this report. The procedure is rapid, sensitive, and reproducible. The reactivities of these preparations were verified on cryostat-cut tissue sections by an IIF assay with rabbit anti-*S. pyogenes* serum. Immunofluorescent staining was concentrated at the basement membranes of kidney and at the sarcolemmal sheaths of cardiac muscle fibers. Immunological cross-reactivity between *S. pyogenes* cells and human or monkey tissue components could not be demonstrated during these experiments by IIF procedures (28).

The adsorption of radiolabeled streptococcal protein to cardiac muscle components appeared to involve high-affinity, specific binding interactions. Binding was time dependent and followed saturation kinetics. Tissue-bound bacterial protein was not significantly desorbed by washing with buffer solutions but was competitively desorbed by nonradiolabeled bacterial components. Specific tissue-binding factors have also been detected on the surfaces of other bacteria (16, 24). The fimbriae (pili) of many gram-negative bacteria exhibit lectin-like binding specificities for carbohydrate moieties on host cell surfaces and appear to be responsible for bacterial adherence (24). Although streptococci are not known to produce true fimbriae, many species possess a feltlike surface of densely distributed fibrils (9, 10). Adherence of these bacteria to host cells (15, 16) and to solid surfaces (20, 27) appears to involve cell wall proteins, or substances closely associated with them, since pretreatment of the bacteria with proteases prevents this phenomenon. Almost nothing is known of the biochemical and immunochemical

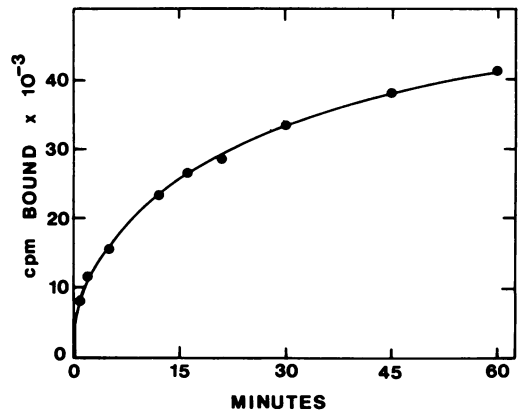

FIG. 3. Rate of binding of ^3H-labeled streptococcal components (1 mg/ml) to cardiac muscle fragments. Binding reactions were terminated at the indicated times by rinsing the tissue fragments with PBS. The radioactivity bound to the tissue components was determined by liquid scintillation spectrometry.

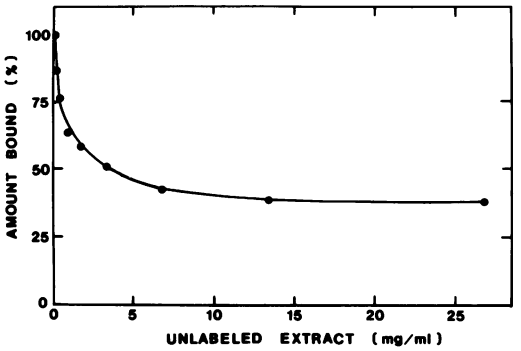

FIG. 4. Competitive desorption of ^{3}H-labeled streptococcal components from cardiac muscle fragments with nonradiolabeled streptococcal components. ^{3}H-labeled bacterial components were incubated with tissue fragments for 60 min. The indicated amounts of unlabeled extract were added, and the incubation was continued for an additional 60 min. The amount of radioactivity remaining on the tissue fragments was then determined.

properties of these adherence-mediating proteins of streptococci.

Lipoteichoic acids of streptococci have been reported to bind to membranes of epithelial cells (1, 4), erythrocytes (3, 8), lymphocytes (2), and heart and kidney cells (12). Removal of esterified fatty acids from lipoteichoic acid by mild alkali hydrolysis destroys its membrane-binding activity (23). In the present study, treatment of the ammonium sulfate-purified bacterial components at pH 10 for 18 h destroyed its erythrocyte-binding activity, as determined by passive hemagglutination with anti-*S. pyogenes* and anti-*Lactobacillus casei* sera, but not its heart- and kidney-binding activities. Moreover, anti-*L. casei* lipoteichoic acid antibodies did not react with the *S. pyogenes* antigen bound to cardiac and kidney tissue (28) but reacted strongly with erythrocytes sensitized with streptococcal lipoteichoic acid. Polysaccharides and teichoic acids extracted from group A streptococci by hot aqueous phenol did not bind to kidney basement membranes or to the sheaths of cardiac muscle fibers (28) as determined by IIF staining. Therefore, lipoteichoic acid does not appear to be the streptococcal component that was bound to monkey tissue in the present study.

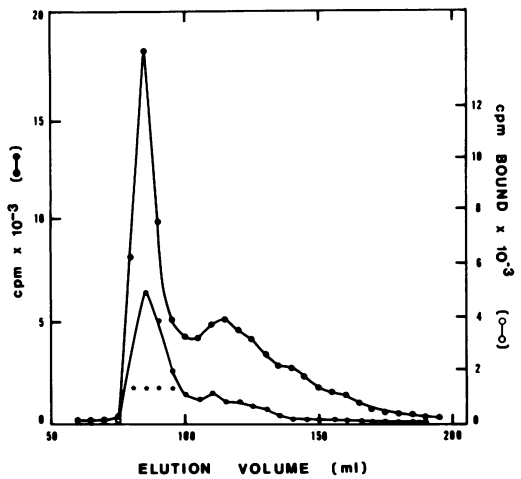

FIG. 5. Separation of ^{3}H-labeled streptococcal components by Bio-Gel P300 column chromatography. Radioactivities of column fractions (●), heart-binding activities determined by direct binding assay (○), and kidney-binding activities determined by IIF assay (*).

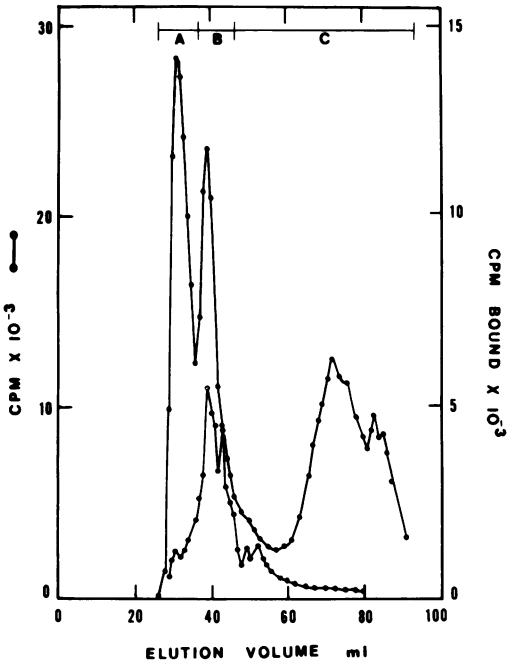

FIG. 6. Separation of ^{3}H-labeled streptococcal components by Sephacryl S200 column chromatography in the presence of 0.1% SDS. Radioactivities of column fractions (●) and heart-binding activities determined by direct binding assay (○).

The contribution of tissue-binding factors in the pathogenesis of streptococcal infections is not known. If they are components of cell wall fibrils, they may mediate the attachment of bacteria to host cell surfaces, which is the first step in many infections (16). It is also possible that tissue-binding factors are involved in the pathogenesis of rheumatic fever or poststreptococcal glomerulonephritis (22, 34). Specific binding factors, released by bacteria growing on the oral mucosa, may enter the bloodstream and accumulate in target organs by direct binding interactions. Histopathology may then be elicit-

ed by one or more of the following events: the binding of the bacterial component may impair function of the tissue component; the tissue-bound bacterial component could activate complement or other mediators of inflammation; the tissue-bound component may combine in situ with circulating antibodies specific for streptococcal antigen; or binding of streptococcal components to tissue may alter host components making them immunogenic, ultimately leading to autoantibody formation.

Cell extracts and cell wall fragments of group A streptococci have been reported to contain antigens that are deposited in cardiac tissue of experimental animals and cause tissue injury (7, 11, 17, 25). The identities and characteristics of the substances responsible for these effects are unknown, since partially purified materials were used in these studies. Streptococcal antigens have also been detected in the glomeruli of patients with acute poststreptococcal glomerulonephritis (19, 29). Lange et al. (19) showed that one of these antigens was also present in whole-cell extracts from a variety of group A streptococci. Villarreal et al. (29) recently described a 46,000-molecular-weight protein that was secreted into the culture medium by nephritogenic strains but not by other strains of group A streptococci. This antigen was also detected in kidney biopsies of acute poststreptococcal glomerulonephritis patients with early symptoms. It is not clear whether these streptococcal antigens were bound directly to kidney components or were deposited nonspecifically as complexes with immunoglobulins. Additional biochemical and immunochemical studies are necessary before the relationship of these nephritis strain-associated antigens and the tissue-binding protein reported in this communication can be defined.

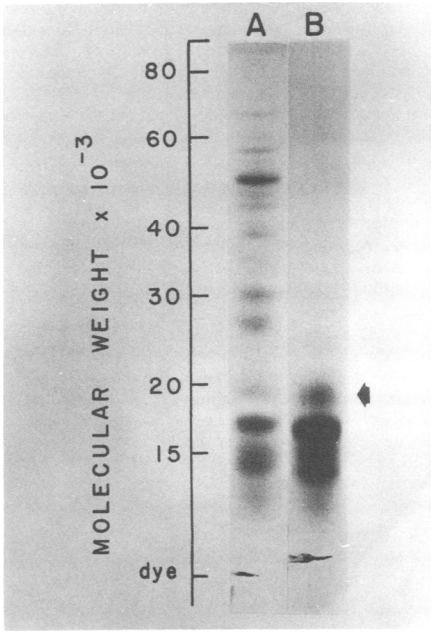

FIG. 7. SDS-polyacrylamide disc gel electrophoresis (10%) of streptococcal extracts. Gel A, 80 μg of crude extract; gel B, 30 μg of peak B material from Sephacryl S200 chromatography. Gels were stained for protein with Coomassie blue. Heart-binding activity was associated with the region indicated by the arrow.

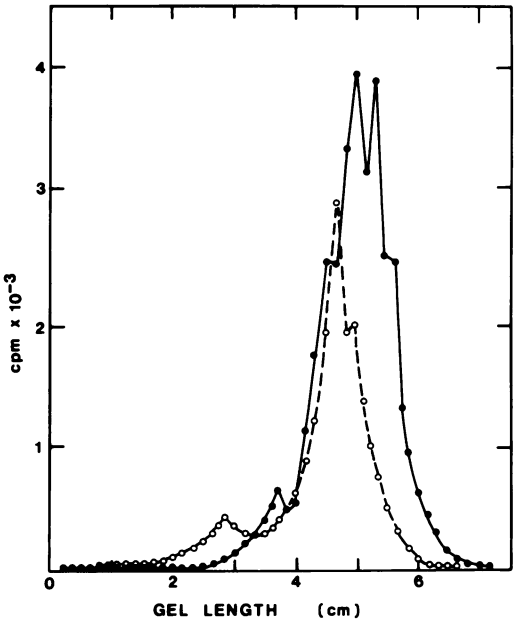

FIG. 8. SDS-polyacrylamide disc gel (10%) electrophoresis of ^{3}H-labeled streptococcal components. Cylindrical gels were sliced into 2-mm sections, and their radioactivities were counted. Partially purified heart-binding factor from peak B of Sephacryl S200 chromatography (●); streptococcal component desorbed from cardiac muscle (○).

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