GROUP A STREPTOCOCCAL ANTIGENS CROSS-REACTIVE WITH MYOCARDIUM

Purification of Heart-Reactive Antibody and Isolation and Characterization of the Streptococcal Antigen*

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It is now well established that when experimental animals such as the rabbit are inoculated with group A streptococci, an antibody appears which binds to heart tissue (1–3). This heart-reactive antibody (HRA)[1] will also bind to skeletal muscle and smooth muscle of vessel walls as well as preparations of sarcolemmal sheath of cardiac myofibers.

HRAs have also been described in the sera of patients with acute rheumatic fever (ARF) (4, 5). Recently, it has been demonstrated that both group A streptococcal cell walls (6) and membranes (7) can absorb these antibodies from the sera. HRA is also present in the sera of patients with postpericardiotomy syndrome (8, 9), Chagas' disease (10), and Dressler's syndrome (11) as well as at the time of cardiac transplant rejection (12). Streptococcal components, however, do not remove the HRA from sera of patients with this latter group of disorders.

The antibodies to heart tissues in the patients with ARF and the detection of deposits of immunoglobulin and complement in the myocardium of patients with rheumatic carditis (13) suggests a possible autoimmune pathogenesis (14). This antibody occurs in these patients at a level fourfold greater than that observed in the sera of patients with uncomplicated streptococcal infections (5) and remains in the sera at elevated titers for a period of 3–5 yr after an acute attack.

A number of attempts have been made by investigators to look further at the streptococcal antigen(s) responsible for this cross-reaction by using hydrolytic methods such as heating to 100°C at pH 2 or pepsin digestion (3, 7). By using his isolated antigen, Kaplan (3) could absorb HRA from the sera of rabbits inoculated with group A streptococci. By means of fluorescent absorption studies, he defined two cross-reactive systems in the cell wall of the streptococci. One was serotype specific while the other was universal to all

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[1] *Abbreviations used in this paper*: ARF, acute rheumatic fever; BSA, bovine serum albumin; CRA, cross-reactive antigen; DEAE, diethylaminoethyl; DNase, deoxyribonuclease; DTT, dithiothreitol; Fl-GAH, fluoresceinated goat anti-human gamma globulin; Fl-GAR, fluoresceinated goat anti-rabbit gamma globulin; HRA, heart-reactive antibody; NTSM, nontype-specific M; PBS, phosphate-buffered saline; RNase, ribonuclease; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SDS-PAGGE, sodium dodecyl sulfate polyacrylamide gradient gel electrophoresis.

group A streptococci tested (6, 15). The antigen preparation was not well characterized other than that it was sensitive to proteolytic enzymes and that it co-purified with type-specific M protein.

Lyampert et al. (16) and Danilova (17) also described a type-specific cross-reactive antigen (CRA) in the cell wall of the group A streptococcus. These antigens cross-reacted only with cardiac myofibers. On the other hand, Zabriskie and Freimer (7) described a CRA which was present in all group A streptococcal membranes. HRA produced against these membranes bound to the sarcolemma of cardiac myofibers and also skeletal muscle and smooth muscle cells of blood vessels. They also demonstrated the presence of a similar antigen in some group C and G streptococci. Nakhla and Glynn (18) demonstrated an antigen in groups A and G streptococci which cross-reacted with cardiac and skeletal tissue. They were able to detect this antigen in an M-negative mutant as did Zabriskie and Freimer (7).

In this report, we present the partial purification from the group A streptococcal membrane of the antigen that cross-reacts with heart. In the course of these studies, it was found that HRAs in antisera of rabbits immunized with group A streptococci differ from those in sera of ARF patients. Neither will inhibit the binding of the other to the sarcolemma of cardiac myofibers, indicating that the antibodies are directed to different antigenic determinants. Accordingly, it proved necessary to purify and characterize HRAs from ARF sera to provide a reagent suitable for monitoring the purification of the relevant streptococcal membrane antigen.

Materials and Methods

Cultures. Streptococcal strains were obtained from the collection of Dr. R. C. Lancefield of our laboratory and were passed monthly through normal human blood. The two group A type 6 streptococcal strains S43/192/2 (mouse passed) and D471 (non-mouse passed) were both originally human isolates.

All organisms were grown in dialyzed Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) prepared as follows: Todd-Hewitt (1,800 g) and yeast extract (Difco Laboratories) (180 g) were suspended in 1 liter of distilled water by heating at 100°C. Antifoam (Dow Corning Corp., Midland, Mich.) was added to prevent excessive foaming. The solubilized media was then placed in dialysis tubing (Fisher Scientific Co., Pittsburgh, Pa.) and dialyzed against two changes of 30 liters distilled water. The dialysate was finally sterilized by filtration into a Biogen Fermentor (American Sterilizer Co., New York) through a 0.22-μm Millipore Filter (Millipore Corp., Bedford, Mass.). The Biogen Fermentor was then inoculated with 1.5 liters of a culture in the logarithmic phase of growth. When the pH of the culture fluid dropped to 7, 2 liters of 50% glucose was added, and the pH was kept constant at 7 by the addition of 5 N sodium hydroxide by using a Radiometer model 11 titrator (Radiometer Corp., Copenhagen, Denmark). Cultures were harvested by using a Sharples high speed centrifuge (Sharples-Stokes Div., Pennwalt Corp., Warminster, Pa.) and frozen at −70°C until processed further.

Preparation of Streptococcal Membrane

GROUP A—PROTOPLAST MEMBRANE. Phage-associated lysin was prepared and purified through the cellulose phosphate chromatography according to the procedure of Fischetti et al. (19). Group A streptococcal membranes were prepared by using the purified group C phage-associated lysin as described by Zabriskie and Freimer (7). After thawing, the organisms (120 g wet weight) were washed with saline and resuspended in 1,200 ml of 0.06 M sodium phosphate buffer (pH 6.1) containing 4% sodium chloride and 5×10^{-4} M dithiothreitol (DTT). To this was added 1.2×10^6 U of DTT-reactivated lysin and 5 mg of deoxyribonuclease (DNase) (Sigma Chemical Co., St. Louis, Mo.). The mixture was incubated at 37°C for 2 h and monitored for complete protoplast formation. After what appeared to be 100% conversion of streptococci to protoplasts, the suspension was incubated for an additional 1 h. The protoplasts were then lysed in hypotonic buffer in the presence

of ribonuclease (1.5 Kunitz U/ml) (RNase) (Sigma Chemical Co.) and deoxyribonuclease (36 Kunitz U/ml) (DNase) (Sigma Chemical Co.). After the cytoplasmic constituents were removed, the protoplast membranes were retreated with the enzymes followed by extensive washes with 0.01 M phosphate-buffered saline pH 7.6 (PBS). The remainder of the treatment and procedures followed those of Zabriskie and Freimer (7) by using 1,200 ml for all volumes. The final protoplast membrane preparations were lyophilized, the final yield usually being approximately 3 g.

GROUP D MEMBRANE. Group D membranes were prepared by glass bead disintegration in a Vibrogen cell mill (Rho Scientific, Commack, N. Y.) according to the procedure of Bleiweis et al. (20) with the modification of van de Rijn et al. (21).

All membrane preparations used in these studies were assayed for contaminating cell walls by using rhamnose as the marker sugar, and only those preparations with less than 0.1% contamination were used in these studies (22).

Preparation of Human Heart Sarcolemmal Sheaths. Fresh heart tissues (10 g) were homogenized in a Sorvall Omnimixer (DuPont Instruments, Sorvall Operations, Newtown, Conn.) in 0.05 M $CaCl_2$ for 5 min at 4°C. The thick slurry was centrifuged at 14,000 *g* for 30 min, and the pellet containing the sarcolemmal sheaths was then washed three times in saline. The sarcolemmal sheaths were then allowed to autolyse in a 100-fold (vol/wt) excess of distilled water for 24 h at 4°C. The sheaths were sedimented at 14,000 *g* for 30 min, and the process was repeated three times. The isolated sheaths were then treated with DNase and RNase at 37°C for 3 h and subsequently washed three times with distilled water and lyophilized. Under light microscopy, very few Z bands remained, indicating the loss of intracellular contents (23). This sarcolemmal sheath preparation served as the source of the insoluble antigenic matrix.

Enzymatic Digestion

TRYPSIN. (bovine pancreas type 3, Sigma Chemical Co.). Membranes (1 mg/ml) were suspended in 0.067 M phosphate buffer (pH 7.5) and incubated at 37°C for 1 h with trypsin (100; 1,000; or 10,000 U/ml). The reaction was stopped by adding twofold excess of soybean trypsin inhibitor (Sigma Chemical Co.).

PEPSIN. (hog stomach mucosa, Sigma Chemical Co.). Membranes (1 mg/ml) were suspended in 0.01 M glycine-HCl buffer (pH 2.2) and incubated at 37°C for 1 h with pepsin (100; 1,000; or 10,000 U/ml). The reaction was stopped by neutralizing the reaction mixture with 0.1 N sodium hydroxide to pH 8.0.

PAPAIN. (papaya latex, Sigma Chemical Co.). Membranes (1 mg/ml) were suspended in 0.1 M sodium phosphate buffer pH 7.0 with 0.01 M cysteine and 0.001 M EDTA and incubated at 37°C for 1 h with papain (10, 20, or 40 U/ml). The reaction mixture was stopped with 0.06 mg/ml iodoacetamide (Sigma Chemical Co.).

PHOSPHOLIPASE-C. (CL perfringens, Worthington Biochemical Corp., Freehold, N. J.). Membranes (1 mg/ml) were suspended in 0.1 M sodium phosphate buffer with 0.01 M calcium chloride (pH 7.3) and incubated for 1 h at 37°C with phospholipase C (5, 25, or 50 U). The reaction was stopped with 10 ml of 0.5 M EDTA (pH 7.0).

ALPHA-GLUCOSIDASE. (*Saccharomyces cerevesiae*, Sigma Chemical Co.). Membranes (1 mg/ml) were suspended in 0.05 M sodium phosphate (pH 6.8) and incubated at 37°C for 1 h with alpha-glucosidase (5, 50, or 100 U/ml).

BETA-GLUCOSIDASE. (Almonds, Sigma Chemical Co.). Membranes (1 mg/ml) were suspended in 0.05 M sodium acetate buffer (pH 5.0) and incubated at 37°C for 1 h with beta-glucosidase (1 or 25 U/ml).

BETA-GALACTOSIDASE. (*Escherichia coli*, Worthington Biochemical Corp.). Membranes (1 mg/ml) were suspended in 0.1 M sodium phosphate buffer (pH 7.0) and incubated at 37°C for 1 h with beta-galactosidase (10, 100, or 1,000 U/ml).

MIXED GLYCOSIDASES. (minimum 12 glycosidic enzymes, Miles Laboratories Inc., Miles Research Products, Elkhart, Ind.). Membranes (1 mg/ml) were suspended in either 0.05 M sodium acetate buffer (pH 5.0) or 0.05 M sodium acetate buffer (pH 6.0) and incubated at 37°C for 18 h with mixed glycosidases (0.2%).

After the above enzymatic digestions were terminated, the residues were removed by centrifugation at 125,000 *g* for 2 h at 4°C. The residues were washed two times with PBS (pH 7.6) and brought to their original volume. The supernate was dialyzed extensively against PBS. These materials were then tested for their ability to absorb HRA.

Lipid Extraction and Fractionation

METHOD I. Membranes (500 mg) were suspended in 10 vol (wt/vol) of chloroform/absolute methanol (2 to 1) (vol/vol) as determined by Folch et al. (24) for the extraction of lipids. The membranes were stirred at 4°C for 15 min. The suspension was then filtered in a Buchner funnel under vacuum (no. 576 filter paper, Schleicher and Schuell), and the extraction procedure was repeated two times. The filtrates were pooled and treated with 0.2 vol of calcium chloride (0.04%). The mixture was shaken vigorously and then allowed to separate into two layers. The upper layer was removed by aspiration, and the two layers were blown dry under nitrogen. The lipids from the lower layer were resuspended in a mixture of chloroform/methanol/water (60:30:4.5), and any other nonlipid contaminants were removed by Sephadex chromatography according to the procedure of Wells and Dittmer (25). After the sample was loaded onto the column, the lipids were eluted by using chloroform/methanol (2:1). Nonlipid material was eluted by using methanol/water (1:1). The lipids and nonlipid eluates were then dried under nitrogen. Lipids were further fractionated on a silica gel column (Sigma Chemical Co., SI L-LC). Lipids were resuspended in 10 ml of chloroform/methanol (9:1) and eluted by using subsequent 75 ml chloroform, acetone, and methanol steps. The various fractions were then blown dry under nitrogen and resuspended in 0.05 M Tris-HCl buffer (pH 8.3) and used for absorption studies. Thin-layer chromatography demonstrated that the chloroform eluate consisted of fatty acids, glycerides, and glycerol whereas the acetone phase contained the glycolipids and the methanol phase the phospholipids.

METHOD II. Membranes were resuspended in ethanol/ether (3:1) and stirred for 15 min at 4°C. The supernate was removed by filtration and the residue was extracted two more times, as above. The filtrates were then combined and blown dry under nitrogen. The residue and extracted lipids were treated as Method I and used for their ability to absorb HRA.

Teichoic Acid Preparation. *Streptococcus mutans* strain BHT wall teichoic acid was prepared by Vaught and Bleiweis (26), group A streptococcal cell wall teichoic acid was prepared by McCarty (27), and erythrocytes sensitized with group A lipoteichoic acid were prepared by Ofek et al. (28). The above preparations were gifts from the individual investigators.

Group A streptococcal lipoteichoic acid was prepared according to the chloroform/methanol/water extraction procedure of Wicken et al. (29) by using protoplast membrane as starting material.

Cardiolipin (Sigma Chemical Co.) was deacylated according to the method of Wilkinson (30).

Gel Filtration Chromatography. Columns were calibrated by passing through them proteins of known molecular weight (bovine serum albumin, ovalbumin, and cytochrome C), and the distribution coefficient of each protein peak was calculated as described by Andrews (31). Blue dextran and ^{22}Na were used to determine void volume and total volume of the column, respectively. Volumes were determined gravimetrically. Column fractions were tested for protein concentrations by the method of Lowry et al. (32), and radioactivity was determined in a Packard model 3022 gamma spectrometer. (Packard Instrument Co., Inc., Downers Grove, Ill.)

Preparation of Rabbit Antisera. Inocula and inoculation schedules for antisera were prepared according to the method of van de Rijn and Bleiweis (33). New Zealand Red male rabbits were bled for preimmune HRA controls. Only rabbits without HRA present in their sera were used for subsequent inoculation.

Preparation of Fluoresceinated Goat Anti-Gamma Globulin. Serum from either rabbits or humans was collected and dialyzed (three times) against 10 vol of 0.02 M sodium phosphate buffer pH 7.0. The dialyzed serum was then loaded onto a column of DE 52 (Whatman Chemicals, Div. W & R Balston, Maidstone, Kent, England) (2.5 vol/vol serum) equilibrated with the above buffer. Tubes containing only gamma globulin as determined by cellulose acetate electrophoresis (34) were pooled and concentrated by using an Amicon stirred cell with a XM 50 membrane (Amicon Corp., Scientific Sys. Div. Lexington, Mass.). Female goats (35–40 pound) were inoculated with 1.5 mg purified gamma globulin in 1 ml incomplete Freund's adjuvant (Difco Laboratories Detroit, Mich.) into their back thigh muscles. Similar injections were administered 3 and 5 wk after the first inoculation. The goats were bled 1 wk after the final inoculation, and the gamma globulin fraction were isolated from the serum as outlined above. The isolated goat gamma globulin fraction was next adjusted to a concentration of 10 mg/ml with 0.5 M sodium carbonate buffer pH 9.5. Fluorescein isothiocyanate (BBL, Cockeysville, Md.) was then added slowly with stirring at 4°C to a final concentration of 14 μg/mg of protein. The reaction was permitted to go overnight

under nitrogen. Tagged gamma globulin was separated from free fluorescein isothiocyanate by passing over a column of Sephadex G-25 equilibrated with 0.0125 M sodium phosphate (pH 5.7). The conjugate was then pooled and dialyzed against 0.01 M PBS pH 7.6. Finally, it was aliquoted and stored at −70°C until use.

HRA Assay. Sera were assayed for the presence of HRA by the indirect immunofluorescence staining technique of Zabriskie and Freimer (7) with the following modifications: the dessicated 4-μm frozen normal heart sections were fixed in acetone for 1 min followed by a 5-min wash in 0.01 M PBS pH 7.6. The sections were permitted to air dry and then incubated with various dilutions of the test sera for 25 min at 23°C in a moist chamber. The sections were again washed for 5 min in PBS and allowed to air dry. The sections were then counterstained with the appropriate fluoresceinated antisera and incubated for an additional 25 min. The sections were finally washed in PBS, air dried, and a cover slip applied by using a mixture of glycerol and PBS (6:4). The slides were analyzed by using a Zeiss Universal Ultraviolet microscope (Carl Zeiss, Inc., New York) with BG 12 and 440-μm filters. All slides were graded on a 0–4+ scale with 4+ representing the greatest intensity of staining.

Absorption of HRA. 5 μl of rheumatic sera which gave a 4+ staining at a dilution of 1:10 were added to a microfuge tube containing 45 μm of a dilution of the sample containing the CRA. The contents were mixed and allowed to incubate at 37°C for 2 h and then overnight at 4°C. Just before use, samples were centrifuged for 5 min in a microfuge 152 (Beckman Instruments Inc., Cedar Grove, N. J.), and the supernate was used in the HRA assay. Unabsorbed sera which were incubated for the same periods of time were used at a 4+ staining control. Normal sera were used as negative controls. All dilutions were in 0.01 M PBS (pH 7.6).

Polyacrylamide Slab Gel Electrophoresis. The purification of the CRA was followed by sodium dodecyl sulfate polyacrylamide gradient gel electrophoresis (SDS-PAGGE) by the method of Laemmli (35) by using a discontinuous Tris-HCl buffer system. Electrophoresis was carried out on a 7–15% gradient slab polyacrylamide gel at 25 mA for 18 h or on 14% polyacrylamide slab gels until the indicator dye just reached the bottom of the slab.

Standards (bovine serum albumin, ovalbumin, carbonic anhydrase, and cytochrome C) were run on all gels for molecular weight determination. Samples and standards in 2% sodium dodecyl sulfate (SDS), 5% mercaptoethanol, and 10% glycerol were boiled (2–5 min) in a water bath before loading onto the gels. After electrophoresis, the gels were placed in a solution of 7% acetic acid and 25% isopropanol for 10 min to remove any excess SDS. The process was repeated for two additional washes in this same solution. The gels were then stained by using 0.2% Coomassie Blue in 7% acetic acid with 25% isopropanol on a Gyrotory water bath shaker (model G76, New Brunswick Scientific Co., New Brunswick, N. J.) for 2 h at 45°C. For destaining, the gels were placed in 7% acetic acid and 25% isopropanol and replaced on the shaker for an additional 2 h. Afterwards, the staining solution was removed and fresh destaining solution was added. The gels were permitted to sit in this solution until all the background stain was removed.

Gels containing ^{125}I-labeled samples were run in a similar manner except that the gels were dried according to the procedure of Maizel (36) and exposed to Kodak X-ray film (Eastman Kodak Co., Rochester, N. Y.) (Blue brand, BB54) to determine their radiolabeled bands by radioautography.

Cellulose Acetate Electrophoresis. Zone electrophoresis of serum proteins was run according to the method of Osterland et al. (34). The acetate strips were then fixed with 20% sulfosalicylic acid and stained with 0.25% Coommassie Brilliant Blue R (Sigma Chemical Co.).

Immunoelectrophoresis. Immunoelectrophoresis was performed by a previously described method (33). Serum samples were electrophoresed at 5 mA per slide for 45 min at 4°C. Rabbit anti-human serum (Behring Diagnostics, Woodbury, N. Y.) was then added to the troughs and incubated at 22°C for 24 h and at 4°C for 72 h.

Protein Determination. All protein determinations were by the method of Lowry et al. (32) by using bovine serum albumin as a standard. Whenever nonionic detergent was present in the protein solution, 4% SDS was added to the sodium carbonate solution to prevent precipitation of the nonionic detergent during the assay.

Radioiodination of Molecules and Binding Assays. Gamma globulin preparations were labeled with ^{125}I by the method of Greenwood et al. (37) and unbound iodine was removed by column chromatography by using Sephadex G-50. Membranes (100 μg) were incubated in the presence of

^{125}I-labeled HRA or normal gamma globulin (150,000 cpm). To each tube was also added ^{22}Na (20,000 cpm) as a volume marker in 1% bovine serum albumin (BSA). The tubes were continuously mixed for 2 h at 37°C and for 18 h at 4°C after which each tube was counted. The tubes were then centrifuged in a Microfuge 152 (Beckman Instruments Inc.) for 5 min and an aliquot of the supernate removed. Each tube was then recounted, and the percentage bound was determined according to the procedure of Gotschlich et al. (38). All samples were assayed in triplicate.

Results

Binding Competition Studies Between Rabbit and Human HRA. Rabbits were immunized with membranes isolated from group A type 6 streptococci. After the immunization schedule, the rabbits' sera contained high titers of antibodies which bound the sarcolemma of human cardiac myofibers as determined by the indirect immunofluorescent staining technique.

Frozen sections of normal and rheumatic human heart tissue were prepared and layered with either undiluted immune rabbit sera or a dilution of ARF sera that gave a 4+ reaction. These sections were counterstained with either fluoresceinated goat anti-human gamma globulin (Fl-GAH) or fluoresceinated goat anti-rabbit gamma globulin (Fl-GAR) as recorded in Table I*a*. Neither counterstain showed any nonspecific staining when placed alone on the respective heart sections. The undiluted rabbit serum exhibited a 4+ reaction while the human serum (diluted 1:10) demonstrated a 4+ reaction (homologous systems).

When the heterologous system was tested; namely, with undiluted rabbit serum incubated first with the section followed by the diluted ARF serum and then the anti-human counterstain, similar results were seen (Table I*b*). The ARF serum still exhibited a 4+ staining, indicating that the rabbit HRA did not block its binding. As demonstrated in the table, the rabbit antibody was still present on the section at a high concentration.

Due to the possibility that the rabbit antiserum did not contain enough antibody molecules to cover all the sites on the tissue, the following experiments were performed. After the initial incubation of the rabbit serum, fresh rabbit anti-membrane serum was incubated with the heart sections for a second incubation period before the ARF serum and counterstain were added. Again, no competition for the same antigenic determinant was demonstrated (Table I*c*).

As a second control to determine whether sufficient antibody was present to bind all of the sites, the rabbit serum after the initial incubation period was transferred to a second, fresh heart section and incubated for 25 min. The section was then counterstained with Fl-GAR and a 4+ staining reaction was observed, exactly the same as seen with the first section, indicating that there was an excess of antibody present in the serum.

To further rule out the possibility that the rabbit antibody binds the same sites as the human antibody, all of the above experiments were repeated at 4°C. At this temperature, the normal incubation times were doubled to achieve the same degree of staining. Again, similar results were observed. All of the above experiments were also done in the reverse order to see if the human antibody could competitively block the rabbit antibody. No inhibition of rabbit antibody by human serum was demonstrated.

Purification of HRA from ARF Sera. From the above experiments, it became evident that the antibodies produced in the ARF patient differ from those

TABLE I
Binding Competition Studies between Rabbit and Human HRA

Counterstain	1st serum	2nd serum	Immunofluorescent staining
(*a*)			
Fl-GAR	R*	–	4+
Fl-GAR	ARF	–	0
Fl-GAH	R	–	0
Fl-GAH	ARF	–	4+
(*b*)			
Fl-GAH	R	ARF	4+
Fl-GAR	R	ARF	4+
(*c*)			
Fl-GAR	R × 2	ARF	4+
Fl-GAH	R × 2	ARF	4+

* R, rabbit serum.

elicited in the rabbit by group A streptococcal membrane. To monitor the isolation of the antigen from the group A streptococcus which cross-reacts with human myocardial tissue, experiments were devised to isolate the HRA from the sera of ARF patients. This was desirable since it is well known that human sera normally contain antibodies to other streptococcal constituents.

Sera from patients with acute rheumatic fever were tested for the presence of HRA by the indirect immunofluorescent staining technique. Those sera which contained a titer of 3–4+ at a dilution of 1:10 were pooled (35 bleedings from 15 patients).

The pooled sera were dialyzed three times against 10 vol of 0.02 M sodium phosphate buffer pH 7.0. The sera were then loaded onto a DE-52 column (Whatman, Inc., Clifton, N. J.) pre-equilibrated with the above dialyzing buffer. The gamma globulin was eluted by using the equilibration buffer. A second step of 0.04 M buffer was added after the protein concentration had reached base line. Fractions were tested for the presence of HRA by the indirect immunofluorescent staining technique. The fractions containing HRA were pooled and concentrated by using an Amicon stirred cell (Amicon Corp., Scientific Sys. Div.) with a XM 50 membrane. Finally, sodium chloride was added back to a concentration of 0.15 M.

To 1 vol of premoistened sarcolemmal sheaths was added 2 vol of column chromatographed HRA, and the mixture was allowed to incubate at 37°C for 2 h with gentle stirring. The sarcolemmal sheaths were then centrifuged at 28,000 *g* for 10 min at 4°C. The supernate was saved for further analysis while the pellet was resuspended and washed four times with a mixture of 5% sucrose, 1% BSA, 0.034 M NaCl, 0.0025 M phosphate, pH 6.8 at 4°C according to the method of Edgington (39). Potassium iodide (2.5 M) was dissolved in PBS, and 10 vol of this solution was rotated for 20 min at 22°C with 1 vol of the washed sarcolemmal sheaths containing adsorbed HRA. The preparation was promptly centrifuged and filtered to remove the sarcolemmal sheaths, and the dissociated HRA was concentrated and equilibrated with PBS by ultrafiltration in an Amicon stirred cell (Amicon Corp.) fitted with an XM 50 membrane.

The optimal concentration of potassium iodide was determined as follows. An aliquot (20 μg) of HRA was labeled with ^{125}I and separated from free iodine by Sephadex G-50 chromatography. The labeled antibody was added to unlabeled antibody and the mixture absorbed to isolated sarcolemmal sheaths. After a 1-h incubation period, the sheaths were washed in cold buffer until the counts in the wash stabilized, indicating the removal of unbound proteins. Analysis of an aliquot of sarcolemmal sheaths by immunofluorescence demonstrated the presence of HRA. Aliquots of the washed sarcolemmal sheaths containing HRA were centrifuged and resuspended in PBS with increasing concentrations of potassium iodide and counted in a gamma counter. After a 20-min incubation period, the sheaths were removed by centrifugation and filtration and the extracts were counted. A maximum recovery rate of 85% was reached at 2.5 M potassium iodide. This is in agreement with the work of Edgington (39) doing similar studies with erythrocyte membranes. A second incubation of the sarcolemmal sheaths with 2.5 M potassium iodide released another 5% of the antibody.

Specificity of the Isolated Antibody. When layered on a human heart section, the isolated HRA bound to the sarcolemmal sheath (Fig. 1 a). The specificity of the binding was confirmed since no staining was observed with brain, kidney, or liver tissues. The antibody bound only to heart and skeletal muscle sections. The only noticeable difference in the staining pattern of the purified antibody was that the intensity of staining to skeletal muscle was substantially decreased after the purification procedure. The isolated antibody also no longer bound to smooth muscle cells of the blood vessel walls.

Purity and Activity of the HRA. Cellulose acetate electrophoresis demonstrated the presence of gamma globulin as well as contaminating BSA from the washing buffer. The BSA could easily be removed by rechromatographing the antibody on diethylaminoethyl (DEAE) cellulose. The cellulose acetate electrophoresis did not demonstrate any other protein moiety with an electrophoretic mobility different from the antibody. Immunoelectrophoresis of the isolated antibody against goat anti-human serum demonstrated only a single precipitin arc in the region of gamma globulin.

Finally, to determine the specificity and purity of HRA, an aliquot of the purified HRA was labeled with ^{125}I. After the free iodine was removed, the antibody was incubated with sarcolemmal sheaths and ^{22}Na for 2 h at 37°C then at 4°C overnight with constant rotation. The tubes were then counted and centrifuged at 13,000 *g* for 5 min. An aliquot of the supernate was removed, and the tube was recounted. Calculations to determine the percent of radiolabeled antibody bound to the sarcolemmal sheaths were done according to Gotschlich et al. (38). Group D streptococcal membranes were added to a duplicate set of tubes as a control. The results are tabulated in Table II. The purified HRA bound only to the sarcolemmal sheaths, indicating again the specificity of the reaction. Also, over 99% of the material was active antibody. To rule out the role of an Fc receptor on the sarcolemmal sheaths, normal gamma globulin controls were also run. No binding of the gamma globulin occurred to either the sarcolemmal sheaths or the group D membranes.

Identification of the Classes of Immunoglobulins Present in HRA. The

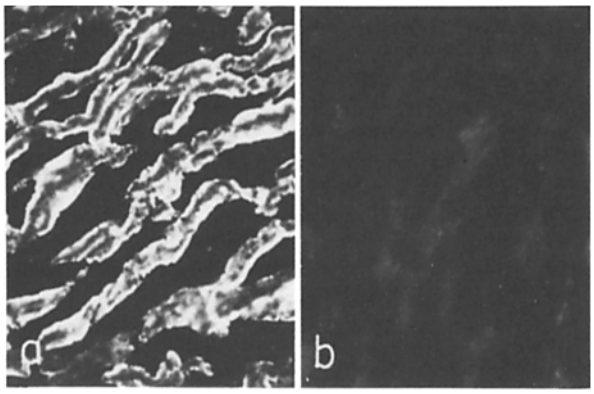

FIG. 1. Immunofluorescent staining of human myocardium with purified HRA isolated from ARF patients. (a) The antibody bound the sarcolemma of the cardiac myofibers. (b) This staining pattern was absent when the HRA was absorbed with purified CRA.

TABLE II
Specificity and Purity of HRA

	Average percent bound*	
	HRA	Normal gamma globulin
Sarcolemmal sheaths	99.4	0.4
Group D streptococcal membranes	2.0	1.3

* All tests were done in triplicate.

purified HRA was layered onto human myocardial sections and counterstained with fluoresceinated heavy-chain-specific antibody. Only antibodies of the IgG class could be demonstrated. This purified HRA was then used to monitor the isolation and purification of the streptococcal CRA.

Distribution of CRA in the Membranes of Various Serotypes of Group A Streptococci. Protoplast membranes were isolated from seven group A streptococcal strains, including six different serotypes, by using purified group C phage-associated lysin. Membranes from a group D streptococcus served as a control. All seven group A streptococcal membrane preparations were able to remove the HRA from acute rheumatic fever serum as demonstrated by loss of fluorescence, indicating the presence of the antigen in the membranes of all types tested (Table III). The group D membrane preparation did not contain the cross-reactive antigen. These results were confirmed by ^{125}I-labeled purified human HRA binding studies. Again, all seven group A streptococcal membrane preparations bound the purified HRA at levels of 97% or greater, whereas the group D membranes bound only 2%. None of the membrane preparations bound more than 3% of the normal human gamma globulin, indicating that nonspecific binding through Fc receptors did not occur in any of the membrane preparations.

Identification of the CRA. To determine the chemical nature of the CRA,

TABLE III
Presence of CRA in Membrane Preparations from Various Serotypes of Group A Streptococci

	Fluorescence‡	Percent binding*	
		Purified HRA	Normal human gamma globulin
Unadsorbed control	4+		
S43/192/2 (type 6)	0	99	2
D471 (type 6)	0	99	1
T12/126 (type 12)	0	97	1
B429 (type 14)	0	100	3
85RP87 (type 18)	0	98	2
D420 (type 41)	±	99	0
A918 (type 55)	1+	97	1
A932 (group D)	4+	2	1

* Membranes (100 μg/ml) were used to bind ^{125}I-labeled purified human HRA or normal human gamma globulin.

‡ Membranes (100 μg/ml) were used to absorb ARF serum. Slides were graded 0–4+ with 4+ indicating the highest intensity of fluorescence.

membranes from group A streptococcal strain S43/192/2 were incubated with various enzymes for a period of 1 h at 37°C. The pellet and supernatant fractions were then assayed for their ability to remove HRAs from ARF patients' sera. Proteolytic enzymes destroyed the antigen as demonstrated in Table IV. Only low concentrations of pepsin (100 U/ml) did not completely destroy the CRA. Lipase and glycosidases had no effect on the antigen nor did they release antigens into the supernate. To further rule out the possibility of a glycoside as part of the immunodeterminant of the CRA, membranes were incubated with a mixture of at least 12 glycosidases at pH 5 and 6 for 18 h with constant mixing at 37°C. Again, there was no loss of activity. Since glycosidases cannot, at times, remove sugar residues from protein due to the short glycosidic chain length, membranes were also treated with 2% periodic acid (not shown) for 1 h at 37°C. Again, there was no loss of activity observed. Even though the involvement of carbohydrates cannot be excluded, the experimental data indicated that the antigen was a protein.

Since lipid extraction of the membrane by the procedure of Folch et al. released a small amount of the CRA (Table V), the various membrane lipids were isolated. The antigen was located in the nonlipid fraction and was destroyed when treated with proteolytic enzymes, giving further evidence that the CRA was a protein. The purified lipid fractions from silica gel chromatography were devoid of the antigen.

Ethanol/ether extraction did not release any of the CRA yet did remove most of the lipid. Upon analysis of the residue, only 5% of the antigen was lost as compared to 10% by using the Folch extraction technique. The ethanol/ether extraction was therefore used as an initial step in purification of this antigen.

Since various investigators (40, 26) have speculated that the CRA was a teichoic or lipoteichoic acid, the following experiments were performed to determine the involvement of teichoic acid as a CRA. Teichoic acid preparations were

TABLE IV
*Effect of Enzymes on Group A Streptococcal Membranes Containing the CRA**

	U/ml	Ability to absorb HRA	
		Residual membranes	Solubilized components
Proteases			
Trypsin	100–1,000	I‡	I
Pepsin	100	A§	I
	1,000	I	I
	10,000	I	I
Papain	10–40	I	I
Lipase			
Phospholipase C	5–50	A	I
Glycosidases			
α-glucosidase	5–100	A	I
β-glucosidase	1–25	A	I
β-galactosidase	10–1,000	A	I
Mixed glycosidases	0.2%	A	I

* Membranes (1 mg/ml) were incubated for 1 h at 37°C, after which they were treated with enzyme inhibitors.
‡ Inactive.
§ Active.

TABLE V
Examination of Various Lipid Fractions from Group A Streptococcal Membranes for the Presence of the CRA

Fraction used for adsorption	Intensity of fluorescence*
Unadsorbed control	4+
Streptococcal membrane	0
Folch extract	3+
Calcium chloride treatment	
Upper layer	3+
Lower layer	4+
Sephadex chromatography	
Nonlipids	
Lipids	4+
Silica gel chromatography	
Glycerol fraction, fatty acids, glycerides	4+
Glycolipid fraction	4+
Phospholipid fraction	4+
Residue	0
Ethanol/ether extract	4+
Residue	0

* All slides were graded on a scale of 0–4+ with 4+ demonstrating the highest level of fluorescence.

obtained from various investigators (see Materials and Methods) as well as isolated from group A streptococci in our laboratory. Neither the wall teichoic acid nor membrane lipoteichoic acid of the group A streptococcus could absorb HRAs (Table VI). Erythrocytes were sensitized with group A streptococcal

TABLE VI
Adsorption of HRA from Acute Rheumatic Fever Patients' Sera by Using Teichoic Acid Preparations

Preparation	Intensity of fluorescence*
Unadsorbed control	4+
Group A streptococcal membrane	0
Group A streptococcal wall teichoic acid	4+
Group A streptococcal lipoteichoic acid	4+
Erythrocytes sensitized with group A streptococcal lipoteichoic acid	4+
S. mutans strain BHT wall teichoic acid	4+
Cardiolipin	4+
Deacylated cardiolipin	4+

* All slides were graded on a scale of 0–4+ with 4+ demonstrating the highest level of fluorescence.

lipoteichoic acid to determine if insolubilized lipoteichoic acid was effective in removing HRA, again, the antibody was not removed. Also, a wall teichoic acid from *S. mutans* strain BHT which is substituted with di- and trigalactosyl residues had no effect on the removal of HRA. Cardiolipin as well as its deacylated derivative also did not remove HRA. This data indicated that the teichoic and lipoteichoic acids were not part of the CRA.

Purification of the CRA. A frequently used method for demonstrating the complexity and diversity of membrane proteins in SDS-PAGGE. Fig. 2 shows the resolution of the total membrane proteins of strain S43/192/2 (type 6) electrophoresed on a 7–15% polyacrylamide gradient slab and stained with Coomassie Blue. Under these conditions, a minimum of 60 polypeptides can be resolved, ranging from a minimum of approximately 10,000 daltons to two high molecular weight polypeptides which just enter the running gel under these conditions. Essentially the same polypeptide distribution can be obtained if 2-mercaptoethanol is omitted from the sample, indicating that there are few, if any, intermolecular disulfide bridges in the proteins of this membrane.

Since the studies indicated that the CRA was a protein, protoplast membranes (1 g) were delipidated by using the ethanol/ether technique. The organic solvents were removed by desiccation of the residue in vacuo. The residue (5 mg detergent/mg residue) was then treated with 4% Emulphogene BC 720 (a gift from GAF Corp., New York) in 0.01 M ammonium bicarbonate buffer (pH 8.3) for 3 h at room temperature with stirring. Particulate material was removed by centrifugation at 125,000 *g* for 2 h at 4°C. Pellets were re-extracted until all of the CRA was solubilized (four to six times) as determined by adsorption. Extracts were pooled and dialyzed against DEAE-cellulose chromatography starting buffer at 4°C overnight.

An aliquot of the extracted material was removed, and the protein was labeled with ^{125}I by the chloramine T method of Greenwood et al. (37). The labeled material, free of unbound iodine, was added back to the dialyzed extract. The dialyzed extracts were next loaded onto a DEAE-cellulose column (1.5 × 20 cm)

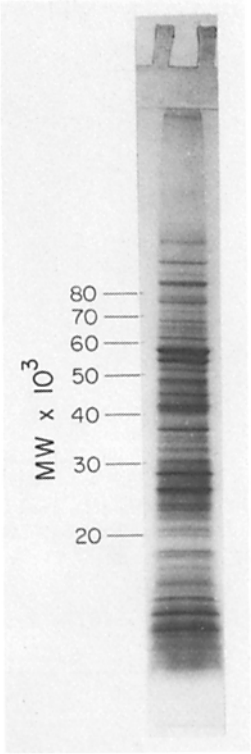

FIG. 2. SDS-PAGGE of SDS-solubilized group A streptococcal protoplast membrane (strain S43/192/2). Sample (150 μg) was loaded, electrophoresed, and stained as outlined in Materials and Methods. A minimum of 60 polypeptides can be discerned from the gel (MW, molecular weight).

which was pre-equilibrated with 0.01 M ammonium bicarbonate buffer (pH 8.3). The column was then washed with 5 vol of starting buffer. The antigen was eluted by using a linear gradient of 300 ml of ammonium bicarbonate (0.01–0.25 M, pH 8.3). A final 1 M ammonium bicarbonate step (pH 7.7) was used to elute the remainder of the protein bound to the column.

Material in the fallthrough, 1 M ammonium bicarbonate step, and the fractions from the linear gradient were assayed for ^{125}I and CRA. The CRA eluted at an ammonium bicarbonate concentration of 0.05 M (Fig. 3) and was not found in any of the other fractions. SDS-PAGE (14%) demonstrated that these fractions contained six polypeptides (mol wt 20–35,000) and some lower molecular weight protein staining material. CRA was purified 65-fold through this step with a yield of 79% (see Table VII).

The fractions containing the antigen were pooled and concentrated by using an Amicon stirred cell (Amicon Corp.) with a UM 10 membrane and chromatographed on a reverse flow Sephadex G150 column (1.5 × 90 cm) equilibrated with 0.2 M ammonium bicarbonate (pH 7.7) and eluted at a flow rate of 10 ml/h. Fractions (1.5 ml) were collected and assayed for their ability to absorb HRA. CRA eluted in fractions 40–55 as shown in Fig. 4. Upon analysis, CRA were shown to be purified 120-fold with a 42% yield (Table VII). The active fractions

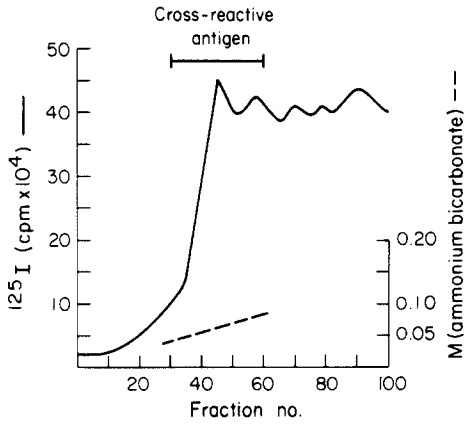

FIG. 3. Elution profile from DEAE-cellulose chromatography of nonionic detergent solubilized streptococcal membrane (701 mg). CRA was eluted by using a linear gradient of 300 ml of ammonium bicarbonate (0.01–0.25 M, pH 8.3). 3-ml fractions were collected at a flow rate of 25 ml/h and monitored for ^{125}I-labeled protein, ionic strength, and their ability to adsorb HRA. The CRA eluted at an ionic strength of 0.05 M.

TABLE VII
Purification of CRA

Fraction	Protein	Activity*	Specific activity	Purification	Yield
	mg	*U*	*U/mg protein*		*%*
Protoplast membrane	740	50,000	67.6	–	100
Ethanol/ether residue	701	45,565	65	–	91
DEAE-cellulose chromatography	8.99	39,500	4394	65	79
Sephadex G150 chromatography	2.6	21,091	8112	120	42

* 1 U of activity is equal to the minimum amount of CRA needed to absorb HRA from ARF sera (50 μl) as determined by loss of fluorescence from 4+ to 0.

were pooled, concentrated, and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) radioautography (Fig. 5). Only four closely-spaced polypeptides could be demonstrated in the pooled, active Sephadex G150 fraction. Analysis of the mol wt of these peptides demonstrated that they were 32,000, 28,000, 26,000, and 22,000 daltons, respectively (Fig. 5). Our final product, then, is composed of four polypeptides which comprise less than 0.6% of the dry weight of the starting material. Finally, the 120-fold purified CRA was incubated with purified HRA to assay for the absorption of HRA. As can be seen by the complete loss of immunofluorescence (Fig. 1 b), the purified CRA absorbed the HRA. This is in comparison to Fig. 1 a in which the HRA was not absorbed.

Discussion

Our evidence suggests that the antibody induced in rabbits by streptococcal membranes is different from that found in ARF patients. The data suggest that the antibodies are directed toward different antigenic determinants on either

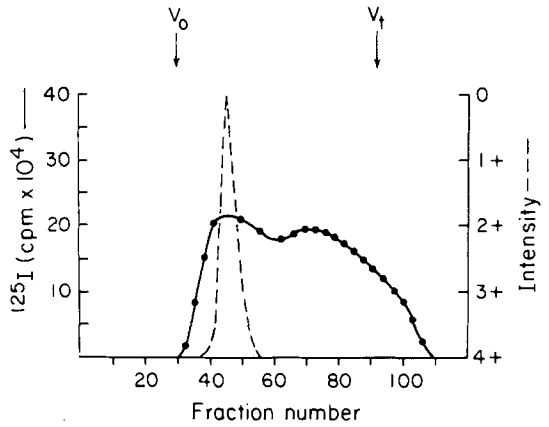

FIG. 4. Elution profile of CRA from Sephadex G150, equilibrated, and eluted with 0.2 M ammonium bicarbonate pH 7.7. A total of 8.99 mg of active pooled fractions from DEAE-cellulose chromatography was applied to the column at an upward flow rate of 10 ml/h. 1.4-ml fractions were collected and analyzed for ^{125}I-labeled protein and their ability to absorb HRA. Intensity is the amount of fluorescence remaining after absorption of HRA with the appropriate fraction.

the same or different molecules. Support for these different antigenic determinants comes from our observations that the intensity of staining by the rheumatic serum is not reduced after preincubation with rabbit anti-heart antibody. The converse is also true; that is, the rheumatic serum will not block the binding of the rabbit antibody to the sarcolemmal sheath. Yet absolute proof of this statement must await isolation and purification of the sarcolemmal antigens involved.

Since our interest lay primarily in the isolation of CRA responsible for human HRA, efforts were directed towards the isolation and characterization of the human antibody. There were two main reasons for pursuing this course. First, since ARF sera contain high titer antibodies to many other streptococcal components, the isolation of the heart-reactive antigen is necessary to monitor the purification of the CRA of the group A streptococcus. Secondly, since the rabbit antibody appears to be directed toward a different antigenic specificity, only human HRA can be used for this purpose.

The purified HRA did not lose any of its specificity by this purification procedure (Table II). Zabriskie et al. (5) demonstrated that 10% of the patients with ARF contained in their sera an antibody which also bound to the smooth muscle cells of the blood vessel walls. It should be pointed out, however, that antibody to smooth muscle cells of the blood vessel walls and skeletal muscle were not efficiently absorbed with our sarcolemmal sheath preparations.

The potassium iodide technique released the antibody from the sarcolemmal sheaths in a native state without the apparent release of sarcolemmal antigen. Potassium iodide acts as a chaotropic ion by modifying the hydrogen bonding and can therefore disrupt intermolecular interactions (41, 42). Antibody isolated in this manner has remained stable over a period of 2 yr.

The work of Kaplan and Meyeserian (2) and Zabriskie and Freimer (7) demonstrated that there was an antigen present in the group A streptococcus

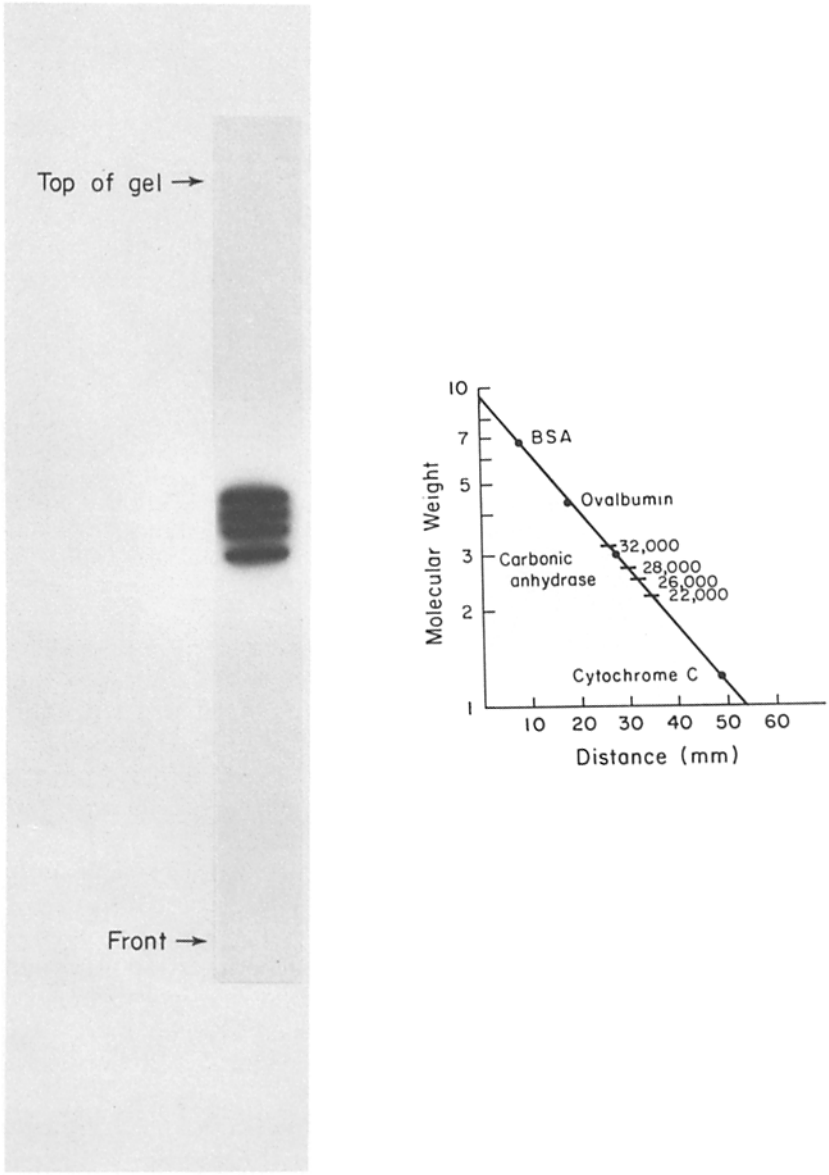

FIG. 5. Autoradiograph of 120-fold purified CRA. A sample of the purified CRA was loaded onto a 14% SDS-PAGE and run as outlined in Materials and Methods. Four polypeptides of 32,000, 28,000, 26,000, and 22,000 daltons, respectively, were resolved.

which was similar to an antigen found on the sarcolemma of the cardiac myofiber. These investigators were able to extract the antigens from the streptococcal membrane or wall by using either dilute hydrochloric acid or pepsin. This type of preparation was able to remove HRA from the serum of ARF patients. Neither group of investigators characterized the antigen to any degree other than to hypothesize that it was a protein since it was trypsin sensitive and that,

in the case of Kaplan (3), the antigen was associated with, but not identical to, the M protein moiety.

Other investigators also have defined immunological systems associated with M protein (43, 44). Beachey et al. (40) defined two non type-specific M (NTSM) associated antigens. One was demonstrated to be a teichoic acid found associated with the cell wall and membrane while the other still remains unidentified. Pachman and Fox (45) and Wittner and Fox (46) also demonstrated that teichoic acid was associated with isolated M protein preparations.

A second NTSM antigen was also defined by complement fixation by Widdowson et al. (44). Protoplast membranes were shown to absorb out these antibodies, and they are present in high titers in ARF patients' sera. Other than this antigen being sensitive to proteolytic enzymes and found associated to some degree with M protein, the antigen was undefined. The relationship of these complement-fixing antibodies to HRAs has not been thoroughly investigated. Finally, Vosti (47) isolated a NTSM antigen from type 12 whole cells and cell walls. Again, its relation to the tissue CRA was not assessed.

In our studies, we have been able to demonstrate that all group A streptococcal protoplast membranes which were tested bind purified human HRA at levels of 97% or greater. Experiments devised to demonstrate that this binding was not through Fc receptors on the membrane indicated that the binding was specific.

Of all the reagents used to determine the chemical nature of the CRA, only proteolytic enzymes could destroy the activity. Earlier work by Zabriskie and Freimer (7) had demonstrated that the antigen could be extracted by using pepsin. Our data indicate that the action of pepsin on the antigen is somewhat limited compared to other proteolytic enzymes but that once the CRA is released from the protoplast membrane, it is easily degraded by the enzyme. Glycosides could not be detected in the antigen preparations. In addition, none of the glycosidases tested were able to destroy CRA, indicating that glycosides were not part of the immunodeterminant. Upon analysis of the lipid fraction, antigen was located in the nonlipid part which was destroyed when treated with proteolytic enzymes. It was later determined that this antigen was identical to that released by using nonionic detergent and that the lipid extraction released only a minor amount of antigen. Finally, our evidence indicates that the CRA is indeed not teichoic acid. None of the streptococcal teichoic acids or cardiolipin could remove HRA. It still remains to be determined whether the antigen is a protein associated with the lipoteichoic acid which is removed from this moiety upon further purification.

SDS-PAGE analysis demonstrated that the group A streptococcal membrane was complex in relation to its protein composition. Of interest was the finding that there are very few, if any, intermolecular disulfide bridges in the proteins of this membrane, a finding which has also been demonstrated in other prokaryotic membranes. Another type 6 membrane from which CRA was isolated (strain D471) contained a very similar polypeptide pattern and had only minor qualitative and quantitative changes when compared to the S43 membrane used in this study.

Extraction of the streptococcal membrane with nonionic detergent released 20–30 polypeptides. It was necessary to extract membranes several times to

remove all of the CRA. Even though the earlier extracts contained different polypeptides than later ones, the antigen was present in all extracts so that we could not disregard particular ones during the extraction procedure. The final preparation of the CRA was purified 120-fold with a yield of 42%. On SDS-PAGE, only four polypeptides were demonstrated by radioautography with mol wt of 22,000, 26,000, 28,000, and 32,000 daltons. Attempts to separate these four polypeptides by isoelectric focusing, affinity chromatography and other forms of ion exchange chromatography have been unsuccessful to date. Because of this, we have been unable to determine the exact location of the immunodeterminant on one or more of these molecules. It is also possible that the immunodeterminant is made up of two or more adjacent molecules. Work is in progress to isolate the individual polypeptides to determine which molecule or molecules contain the active determinant.

Summary

Heart-reactive antibody (HRA) appears in the sera of experimental animals inoculated with group A streptococci as well as patients with acute rheumatic fever. Adsorption of either serum with group A streptococcal membranes will remove the HRA. Blocking experiments between these two types of HRAs have demonstrated that the antibodies are directed towards different antigenic determinants on either the same or different molecules.

To isolate and purify the antigen from the group A streptococcus cross-reactive with sarcolemmal sheaths of cardiac myofibers, it became necessary to purify the HRA from rheumatic fever patients' sera. Isolated gamma globulin containing all of the HRA was adsorbed onto human sarcolemmal sheaths. The specific HRA was released by using potassium iodide. Over 99% of the purified HRA was shown to bind the sarcolemmal sheath whereas less than 1% of the antibody would bind nonspecifically to other material.

Preparations of group A streptococcal membrane will bind HRA purified from the sera of acute rheumatic patients at levels of 97% or greater. The cross-reactive antigen solubilized by nonionic detergent was purified 120-fold by column chromatography. On sodium dodecyl sulfate polyacrylamide electrophoresis, the antigen was demonstrated to be composed of four polypeptides with mol wt of 32,000, 28,000, 26,000, and 22,000 daltons, respectively. Only proteolytic enzymes could destroy the antigenic determinant whereas glycosidases and lipases had no effect. The purified antigen blocked the binding of purified HRA to normal human heart sections.

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