AN IMMUNOLOGICAL RELATIONSHIP BETWEEN THE GROUP A STREPTOCOCCUS AND MAMMALIAN MUSCLE*

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PLATES 75 TO 77

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Although the available evidence clearly indicates that the Group A streptococcus represents the probable etiological agent of acute rheumatic fever, its role in the pathogenesis of this disease remains obscure. One of the widely considered theories suggests that some individuals become sensitized to one or more streptococcal antigens during an infection with this microorganism, and that the acute rheumatic process stems from the interaction between these antigens and antibodies within the tissues of the host. This concept of hypersensitivity has led to many studies of the antigens of Group A streptococci. However, in spite of the accumulation of a large body of information about these antigens, the pathogenesis of rheumatic fever remains an enigma. No specific antigen has been identified, and the manner by which the inflammatory reaction is invoked and sustained is unknown at this time.

In an attempt to determine whether streptococcal antigens or antigen-antibody complexes are present in the lesions of acute rheumatic fever, Coons reasoned that a visibly tagged antibody might be of aid in the localization of streptococcal antigens at the sites of histological damage in rheumatic fever (1). Although Coons turned from his original objective, the immunofluorescent technique which he developed has served as a spring board for many other investigations into the role of antigen-antibody reactions in rheumatic fever.

In the early immunofluorescent studies, investigators examined the involvement of host gamma globulin in the pathological process. In a small series of patients, Vazquez and Dixon noted that gamma globulin was preferentially concentrated in the myocardial lesions of rheumatic fever (2). In a more extensive study by Kaplan, bound gamma globulin was detected in widely scattered sites throughout the myocardium in 20% of a series of surgical specimens obtained from rheumatic hearts (3). In another detailed study, Wagner did not find any relationship between the bound gamma globulin which he demonstrated in the Aschoff bodies of rheumatic hearts and fluorescein-tagged streptococcal antigens. In addition, by using fluorescein-tagged Group A

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antisera he was unable to detect any streptococcal antigens *in situ* in these hearts (4).

As an outgrowth of these studies, Kaplan subsequently reported that a number of the antisera prepared by immunizing rabbits with cell walls of certain Group A streptococci bound to heart muscle, and that protein-containing extracts of these cell walls blocked the binding of this streptococcal antibody (5, 6). He suggested that these cell walls contained a "cross-reactive" protein antigen similar in some chemical features but immunologically distinct from streptococcal type-specific M protein. He also detected antibodies cross-reactive with heart muscle in sera obtained from patients either with recent streptococcal infections or with the nonsuppurative sequelae of streptococcal infection (7).

Studies in our laboratory also provided evidence that antibody to Group A streptococci reacted with cardiac muscle, but this heart-reactive antigen appeared to be more widely distributed among Group A streptococci than the antigen described by Kaplan. When our preliminary experiments indicated that the heart-related antigen was present in the streptococcal membrane, we began a detailed investigation of this antigen-antibody reaction.

This report contains a description of the immunofluorescent staining of cardiac muscle by this streptococcal antibody, and the results of serological studies which showed that this heart-reactive antigen was present in all strains of Group A streptococci and that it was absent in unrelated Gram-positive cocci. Furthermore, chemical and immunological studies localize this streptococcal antigen in the cell (protoplast) membrane of the Group A streptococcus, and suggest that this cross-reaction represents an immunological relationship between the streptococcal membrane and the sarcolemma, the complex membrane structure of striated muscle cells.

Materials and Methods

Strains of Streptococci.—The streptococcal strains used in these experiments were all from The Rockefeller University collection. The other Gram-positive cocci were also from this collection.

Serological Identification.—The serological identification of the strains used in these experiments was confirmed with the capillary tube precipitin test using streptococcal group and type-specific rabbit antisera (8).

Culture Media.—Nutrient Todd-Hewitt and dialysate broths were prepared as previously described (9). In a number of critical experiments, trypticase-soy broth (Difco Laboratories, Inc., Detroit), free of mammalian heart products, was used.

Preparation of Group C Phage-Associated Lysin.—Partially purified Group C phage-associated lysin originally described by Krause (10) and Maxted (11) was prepared using a modification of the procedure of Fox (12). Todd-Hewitt broth was inoculated with Group C streptococcal strain 26RP66. When the bacterial growth at 37°C reached an optical density of 0.07 (measured at 650 mμ in a Coleman Jr. spectrophotometer), a suspension of Group C phage (10^9 PFU/ml), in which the phage-associated lysin had been inactivated by prior storage at 4°C for 2 wk, was added to the bacterial culture. The proportion used was 1 volume of phage suspension to 3 volumes of streptococcal culture.

To prepare a concentrated lysin, the phage-infected bacteria were collected and resuspended in $\frac{1}{100}$ of their original volume before lysis as suggested by Fox. The suspending medium was 0.067 M phosphate buffer (pH 6.0) containing 0.01 M mercaptoethanol and 0.05% magnesium-activated DNAse. Lysis occurred within an hour at 37°C. Cell debris and bacteriophage were sedimented by centrifugation at 35,000 RPM for 1 hr in a No. 40 rotor of a Model L Spinco preparative ultracentrifuge.

The muralytic activity in the phage-free supernatant was assayed in the following manner. Serial dilutions of the lysin were activated with 0.1 ml of 0.01 M mercaptoethanol. The bacteria in an 18 hr culture of Group A streptococcal strain, S43/100, were collected, and resuspended in 0.067 M phosphate buffer (pH 6.0) at an OD of 0.15. 1 ml of the streptococcal suspension was added to each dilution of lysin, and the mixture incubated at 37°C for 1 hr. The end point was the last tube showing complete lysis of the bacteria. The activity in each batch of lysin was quite similar with an end point at a dilution of 1:10,000.

Fifteen-liter lots of active lysin have been prepared by this method using a Sharples centrifuge for collecting the phage-infected organisms. The lysin was stored at −70°C, either in a CO_2 box or in a mechanical refrigerator.

Preparation of Streptococcal Membranes.—Streptococcal protoplast membranes were prepared by the method previously described (13) with the following modification. The partially washed protoplast membranes were reincubated at 37°C with phage-associated lysin in 0.067 M phosphate-buffered saline (pH 6) for 1 hr. This second treatment with lysin insured the removal of the last traces of cell wall carbohydrates from the membranes.

Preparation of Cell Walls.—Cell walls were prepared as previously described (13).

Analytical Methods.—Rhamnose was determined by the method of Dische and Shettles (14). Quantitative glucosamine determinations were done by a modification of the Elson and Morgan procedure (15). Quantitative glucose analyses were done by a modified method employing glucose oxidase (glucostat) avalable from the Worthington Biochemical Corporation, Freehold, New Jersey. Ribonucleic acid was determined by the orcinol reaction. Total nucleic acid content and protein concentrations were determined by the absorption of solutions at 260 and 280 mμ respectively in the Beckman UV spectrophotometer.

Preparation of Immune Sera.—Sheep sera containing antibodies to rabbit gamma globulin were prepared in the following manner. Rabbit gamma globulin, Cohn Fraction II, (Pentex Inc., Kankakee, Illinois) was further purified by column chromatography on DEAE-cellulose, lyophilized, and stored in a desiccator at 4°C. 200 mg of this globulin, dissolved in 20 ml of saline, was emulsified with an equal volume of Freund's incomplete adjuvant, and injected intramuscularly at four separate sites in a sheep. After 3 wk, another 100 mg of globulin was injected subcutaneously, followed by three additional 50 mg injections at 2-wk intervals. 10 days after the last injection, 400 ml of blood were withdrawn. The serum was separated, rapidly frozen, and stored in 30-ml aliquots at −70°C. Rabbits were immunized with highly purified human IgG prepared by the method of Strauss et al. (16). Sera containing antibodies to membranes were obtained by immunizing rabbits with purified protoplast membranes, while rabbit antisera to the other bacterial fractions were prepared as described (8, 13).

Preparation of Fluorescein-Labeled Globulins.—The gamma globulins were isolated from various sera as follows: Each antiserum was dialyzed for 16 hr at 4°C in 0.015 M phosphate buffer (pH 7.1), passed over DEAE-cellulose (Serva), 0.6 meq/g, (Gallard-Schlesinger Chemical Mfg. Corp., Garden City, New York) at 25°C, and then eluted with the same buffer. To prevent overloading the column, it was important not to add more than 0.8 ml of serum to each gram of DEAE-cellulose. The eluted gamma globulin was concentrated by ultrafiltration in S & S collodion bags (Carl Schleicher and Schuell Co., Keene, New Hampshire), and its purity confirmed by cellulose acetate paper electrophoresis.

At 4°C, the solution containing 15 mg of gamma globulin/ml was brought to pH 9.5 by the addition of 0.1 N NaOH. Crystalline fluorescein isothiocyanate (Sylvana Chemical Co., Orange,

New Jersey), 0.03 mg per mg of protein, was added slowly while the globulin solution was stirred continuously. During the first 2 hr of the reaction the pH was maintained between 9.2 and 9.5 by means of a Radiometer type PHM 28 titrator 11, (Copenhagen), with probe electrodes, standardized at pH 4.0. The vessel was then stoppered, and the reaction allowed to proceed for 18 hr without pH control.

Following coupling of the globulin, unconjugated fluorescein isothiocyanate was removed by passage through a Sephadex G-25 column (Pharmacia, Uppsala, Sweden) which had been equilibrated with 0.05 M phosphate-buffered saline (pH 7.5). Each ml of conjugated globulin containing 15 mg of protein, was absorbed with 8 mg of a rabbit liver powder to remove nonspecific fluorescence, and stored at 4°C.

Tissue Specimens.—The tissues used in the immunofluorescent studies were obtained at surgery from individuals with inactive rheumatic heart disease or with congenital heart defects.[1] Biopsy specimens from the left auricular appendages or from ventricular myocardium were quickly frozen to −70°C within 60 min of surgical removal and stored at −70°C in a CO_2 box. Representative sections of frozen as well as formalin-fixed tissues were stained with hematoxylin and eosin.[2] In addition, sections were prepared from biopsies of heart, lung, liver, uterus, spleen, intestine, and synovia obtained either at surgery or at necropsy from patients dying of unrelated diseases.

Immunofluorescent Staining Techniques.—Representative sections cut at 4 or 6 mμ at −20°C in a cryostat, were fixed for 2 min in acetone, and washed twice in 0.05 M phosphate-buffered saline (pH 7.5). After removal of excess moisture, the sections were stained by one of the following methods:

1. Direct technique: Each tissue section was covered with 1 to 2 drops of fluorescein-conjugated globulin, and incubated for 30 min in a moist chamber at 25°C.

2. *Indirect Technique:* Each section was incubated with unlabeled rabbit or human antiserum for 30 min as above. After two 5-min washes with buffered saline, fluorescein-conjugated sheep anti-rabbit, or rabbit anti-human IgG globulin was applied. Then, the section was incubated for an additional 30 min.

Following either procedure 1 or 2, the stained sections were washed twice in phosphate-buffered saline for 5 min each, mounted in glycerol buffered at pH 7.5, and examined. Although minor variations in the intensity and degree of staining were observed, both the direct and the indirect techniques produced essentially the same pattern of fluorescent staining.

Fluorescence Microscopy.—Sections were examined with a Reichert "Biozet" microscope. The ultraviolet light source was a HBO-200 mercury lamp with a red excluding filter. For visualization of fluorescent staining, UGI, OG4, and BG12 filters alone or in combination were used. For photographic records of stained sections, 35-mm black and white film, Adox KB17, and Ansco 200 color film were used.

In crucial experiments, serial photomicrography was employed. Each section was photographed under identical exposure conditions, and contact prints of these serial photomicrographs were used to evaluate the degree and intensity of immunofluorescent staining.

EXPERIMENTAL

The Nature of the Immunofluorescent Staining of Cardiac Muscle with Group A Streptococcal Antibody.—When a section of human heart tissue is incubated

[1] These specimens were kindly supplied by Dr. Fred Bowman, Dr. Ferdinand F. McAllister, and Dr. James Malm of the surgical service of the Columbia Presbyterian Medical Center, New York.

[2] These sections were reviewed by Dr. Robert Lattes and Dr. Nathan Lane of the surgical pathology department of the Columbia Presbyterian Medical Center, New York.

with rabbit antiserum to Group A streptococci, streptococcal antibody is bound to the cardiac muscle cells. If fluorescein-labeled antibody is used, this antigen-antibody reaction can be visualized by fluorescence microscopy. A distinctive pattern of immunofluorescent staining is seen, in which the bright apple-green fluorescence of the muscle fibers stands out in contrast to the blue autofluorescence of unstained connective tissue. Although fluorescent staining patterns are better reproduced in color, good contrast can be obtained in a black and white photograph. One such pattern with diffuse staining of the sarcoplasm is illustrated in Fig. 1. When a serum without heart-reactive antibody is incubated with cardiac tissue, the myofibers remain unstained and appear dark brown (see Fig. 2).

In some sections, such as the one shown in Fig. 3, the cross-striations of cardiac muscle were clearly outlined; at times creating a scalloped effect along the margin of the myofiber. The most intense staining was found in the region of the sarcolemma, the complex membrane structure which surrounds the muscle cell, (see Figs. 4 *a* to 4 *c*). Although this observation suggested that the sarcolemma was the site at which the streptococcal antibody was bound, precise definition could not be obtained at the limit of resolution of fluorescence microscopy.

In our studies of the immunological reactions between streptococcal antibody and cardiac muscle, more than 3000 sections were examined from over 100 different biopsy specimens obtained at surgery. These specimens were from patients with rheumatic or congenital heart disease, but regardless of the origin, both the patterns and the intensity of the immunofluorescent reactions were similar in each section. The fluorescent staining reaction was not limited to cardiac muscle, for specimens of skeletal muscle obtained from a number of different voluntary muscles also bound streptococcal antibody.

In general, smooth muscle cells did not react, but the layer of smooth muscle in the media of the endocardium, and smooth muscle elements in the medial wall of medium-sized arterioles in other areas of the body as well as those in the heart, stained brightly when labeled streptococcal antibody was applied. Staining of such an arteriole in heart muscle is seen in Fig. 5. Although arterioles always stained in sections of such tissues as the synovial lining of joint spaces, the other cellular elements of this tissue as well as those of liver, spleen, and portions of the genito-urinary tract did not react. In contrast to this tissue specificity, the reaction of streptococcal antibody with muscle cells was not species specific. For example, heart sections from normal rabbits or from normal guinea pigs showed the same staining patterns as those from human hearts when they were incubated with fluorescein-labeled streptococcal antibody.

The Distribution of the Heart-Reactive Antigen among Hemolytic Streptococci.— Turning to an investigation of the streptococcal antigen involved in the reaction with heart tissue, a study of its distribution within the various groups of hemo-

lytic streptococci was begun. As the distribution of this antigen among the many serological types of Group A streptococci was of special significance, rabbit antisera to a wide variety of Group A streptococcal types in addition to those representing most of the other streptococcal groups were tested. Antisera

TABLE I

Demonstration of the Heart-Staining Antibody in Antisera to Group A Streptococci

Rabbit antisera to whole cells of	No. of sera	Immunofluorescent reactions		
		No. positive	No. negative	Intensity of staining*
Hemolytic streptococci				
Group A	48‡	47	1§	2+/3+
" A var.	3	3	0	2+/3+
" B	4	0	4	0
" C	6	4	2	1+/2+
" D	4	0	4	0
" E	3	0	3	0
" F	6	1‖	5	+
" G	6	0	6	0
" H	2	0	2	0
" L	1	0	1	0
" M	1	0	1	0
" N	1	0	1	0
Staphylococcus aureus	1	0	1	0
Staphylococcus albus	1	0	1	0
Diplococcus pneumoniae	2	0	2	0
Preimmune serum controls	15	0	15	0

* These results represent the average staining reaction of each antiserum after absorption with rabbit liver powder.

‡ The 48 group antisera were unabsorbed, and represented 21 different serological types as well as two strains, J17A4 and S43 glossy, that had no type-specific M protein.

§ This serum was prepared with strain, B514, originally isolated from a mouse infection.

‖ This serum was prepared with strain H127 isolated from a human throat infection.

to other Gram-positive cocci were used as controls. Since whole killed bacterial suspensions were used for immunization, each serum contained antibodies directed against many of the cellular antigens. However, specific antisera also were prepared to the protoplast membrane and to cytoplasmic materials. The presence of heart-reactive antibody in each serum was determined by both the direct and the indirect fluorescent staining techniques, and the intensity of each staining reaction was graded from 4+ to 0.

The average intensity of immunofluorescent staining reactions between a large series of bacterial antisera and heart sections are recorded in Table I.

These data clearly demonstrate that the heart-staining antibody was present in antisera to Group A and A variant streptococci. Although the intensity varied from one serum to another, fifty unabsorbed Group A and A variant antisera representing twenty-one different serological types produced strong staining reactions. Only one Group A serum, prepared with strain B514 originally isolated from a mouse infection, failed to stain the heart sections. This immunofluorescent reaction evidently did not involve antibody to type-specific M pro-

TABLE II

Immunofluorescent Reactions which Demonstrate the Heart-Staining Antibody in Antisera to Membranes of Group A Streptococci

Rabbit antisera to membranes of	No. of sera	Immunofluorescent reactions		
		No. positive	No. negative	Intensity of staining
Hemolytic streptococci				
Group A	15	15	0	4+
" C	2	0	2	0
" D	3	0	3	0
Streptococcus viridans	2	0	2	0
Staphylococcus aureus	1	0	1	0
Preimmune serum controls	15	0	15	0

tein since a variety of serological types as well as Group A strains that contained little or no M protein produced identical reactions. Furthermore, six antisera prepared by Dr. Eugene Fox to highly purified M protein antigens failed to react with the heart sections (17).

In contrast to Group A antisera, only a few of the other group-specific antisera showed positive reactions. These reactions, mainly with Group C antisera, were weaker in intensity than those of Group A. The antisera to the other groups of hemolytic streptococci and to the other Gram-positive cocci failed to react at all.

When sera prepared by immunization with purified protoplast membranes and sera containing antibodies to other streptococcal cellular components were examined for the presence of heart-staining antibodies, the results were even more striking. All of the antisera to Group A streptococcal membranes produced extremely bright (4+) staining patterns. A similar reaction was noted with specific membrane antibody globulin, obtained by absorbing membrane antisera with streptococcal protoplast membranes and dissociating the globulin from the specific precipitate at pH 3.0. In contrast, antisera to cytoplasmic material, and antisera to membranes from other streptococcal groups as well as

from other Gram-positive cocci did not react. The results of several of these studies are recorded in Table II.

These immunofluorescent studies show that antisera to Group A streptococci contain an antibody which reacts with cardiac tissue, and indicate that this antibody is formed in response to an antigen localized in the membrane of the streptococcal cell.

Specific Absorption Studies Demonstrating the Localization of Heart-Reactive Streptococcal Antigen in the Cell Membrane.—In view of the observations indicating that the heart-reactive antigen resided in the streptococcal membrane, additional experiments were designed to confirm the localization of this antigen. The ability of various streptococcal components to absorb heart-staining antibody from Group A streptococcal antisera and from membrane antisera was studied in a series of experiments.

In a typical experiment using the direct staining technique, a solution containing fluorescein-labeled antibodies to membranes of Group A streptococci was diluted to a concentration that still produced bright staining of representative heart sections. Weighed amounts of antigen were added to 0.2 ml aliquots of this dilute globulin solution, and each mixture was incubated, first at 37°C for 60 min, and then at 4°C for 18 hr. As a control, 0.2 ml of the same globulin solution was incubated without any antigen. After centrifugation, a portion of each supernatant was applied to a heart section. The sections then were incubated, washed, and examined by the methods described above.

In every experiment, the reference point was a section stained with the dilute solution of unabsorbed globulin. The degree of absorption of heart-reactive antibody by a given weight of an antigen was evaluated by comparing the intensity of the fluorescent reaction produced by the absorbed globulin with that of the unabsorbed globulin. An example of quantitative absorption of heart-reactive antibody by increasing weights of antigen is shown in Figs. 6 *a* to 6 *d*. The brightest intensity (4+) of staining (Fig. 6 *a*) was produced by the unabsorbed globulin. Partial absorption of the heart-reactive antibody resulted in decreased staining (see Figs. 6 *b* and 6 *c*) while Fig. 6 *d* shows the absence of staining (0) that resulted when all of the antibody had been removed.

Antigenic fractions were obtained from representative strains of many of the the serological groups of hemolytic streptococci, and from strains of *Streptococcus viridans* and *Staphylococcus aureus* as controls. Lyophilized preparations of cell walls and membranes from each strain, and preparations of cytoplasmic material and whole cells from several strains were examined for the presence of antigens capable of absorbing the heart-reactive antibody.

The results are summarized in Table III. These studies demonstrate the complete absorption of heart-reactive antibody by membranes of Group A streptococci. In most experiments, absorption with as little as 1 mg of purified membrane abolished the staining reaction. Membranes from 36 strains of Group A and A variant streptococci representing fourteen different serological types and

several nontypeable strains, all proved equally effective in removing this antibody. Membranes from only one Group A strain, A236, originally isolated from a mouse infection, failed to block the fluorescent staining reaction.

Although membranes from some strains of Group C and Group G streptococci partially absorbed the immunofluorescence, at least 5 mg of these membranes were necessary to produce 50% inhibition (2+). Membranes from other streptococcal groups, and membranes from other Gram-positive cocci produced no

TABLE III

The Absorption of Heart-Reactive Antibody by Membranes of Group A Streptococci

Antigens prepared from	No. of strains	Absorption with							
		Whole cell		Cell wall		Cell membrane		Cytoplasmic material	
		Weight	Int.*	Weight	Int.	Weight	Int.	Weight	Int.
		mg		*mg*		*mg*		*mg*	
Hemolytic streptococci									
Group A‡	33	10	2+	5	+/2+	1	0	10	4+
" A var.	3	10	2+	5	+/2+	1	0	10	4+
" C	4	10	4+	10	4+	5	2+	10	4+
" D	3			10	4+	10	4+		
" E	1			10	4+	10	4+		
" F	1			10	4+	10	4+		
" G	2			10	4+	5	2+		
Streptococcus viridans	2			10	4+	10	4+		
Staphylococcus aureus	1			10	4+	10	4+		
Unabsorbed globulin control			4+		4+		4+		4+

* Int., intensity of the immunofluorescent staining reaction after absorption.

‡ Group A membranes used were prepared from the following types: 1, 3, 4, 5, 6, 12, 14, 19, 22, 24, 26, 30, and 50 and several nontypeable strains. Of the 33 strains tested, only one, A236, a strain isolated from mice, failed to absorb the heart-reactive antibody.

detectable diminution of the immunofluorescent reaction although the antibody solution was absorbed with as much as 10 mg of each antigen.

While cytoplasmic material from a number of Group A and Group C strains did not decrease the fluorescent staining of cardiac tissue, absorption with either 5 mg of cell walls or 10 mg of whole cells from Group A streptococci significantly reduced the intensity of staining (2+). As the cell membrane represented about 10% of the dry weight of the streptococcus, it seemed likely that the absorption of the heart-staining antibody by whole cells was a reflection of the presence of the cell membrane. This concept was supported by serological evidence which showed that almost all sera prepared against whole cells of Group A streptococci contained membrane antibodies. On the other hand, the absorption of heart-

reactive antibody from membrane antisera by cell walls was perplexing. In view of these findings and those of Kaplan, several immunochemical studies of the cell wall were undertaken to determine the extent to which membranes were present in cell wall preparations.

The Presence of Membranes in all Cell Wall Preparations.—Intensive studies of cell walls of Group A streptococci have focused largely on the group-specific carbohydrate, and more recently on the mucopeptide backbone of the wall (18). Since polysaccharides have represented the major interest, current immunochemical information is derived largely from cell walls which have been modified

TABLE IV

Chemical Analyses which Demonstrate the Presence of Membranes in all Cell Wall Preparations Isolated from Group A Streptococci

Preparation	Glucose	Rhamnose	Glucosamine
	%	%	%
T25	0.33	23.6	20.7
T1/19	0.40	17.6	17.3
T22	0.50	13.0	13.7
T25	0.40	22.4	16.7
T25/41	0.37	25.2	19.2
*T25/41	0.30	28.9	22.0

* After reprocessing.

by such hydrolytic procedures as enzymatic digestion, heat, or treatment with mineral acids.

In previous studies of protoplast membranes a number of cell wall preparations examined by both chemical and immunological techniques were found to contain a considerable amount of membrane material (13). Similar observations subsequently were reported by Schmidt (19). In his studies of the lysis of Group A streptococcal cell walls by the enzymes of *Streptomyces albus*, nontrypsinized cell walls were found to be grossly contaminated with cell membranes. The following experiments were designed to extend these observations and to see whether membrane "contamination" of the isolated cell walls could be reduced.

Cell walls were prepared from a number of strains of Group A streptococci by the usual methods. The results of chemical analysis of five of these preparations are recorded in Table IV. As each preparation contained a considerable amount of glucose, a sugar found in the cell membrane but not in cell wall carbohydrates of Group A streptococci, the walls were resuspended in saline and reprocessed. Chemical analysis of one lot of walls, after reprocessing, is included in Table IV. Although the walls contained a higher percentage of rhamnose and glucosamine after these procedures, it is evident that their glucose content was not significantly decreased.

In another attempt to separate cell walls from membrane fragments, streptococci, disrupted by shaking with glass beads, were centrifuged in sucrose gradients. The chemical composition of the fractions again showed that this method had not separated these two components.

These studies supplied chemical evidence which demonstrated that all of our preparations of Group A streptococcal cell walls contained a considerable proportion of cell membrane; at times as much as 40%. This concept was well supported by a number of serological studies. For example, extracts of cell walls contained antigens which consistently reacted with rabbit antisera to purified

TABLE V

Comparison of the Chemical Composition of the Protoplast Membrane and the Insoluble Residue of Cell Wall Lysis

Preparation	Lot	Weight	Rhamnose	Glucosamine	Glucose
		mg	%	%	%
Cell wall	I	300	22.4	16.7	0.4
Insoluble residue	I	60	0.1	0	0.7
Cell wall	II	250	23.8	14.0	0.3
Insoluble residue	II	72	0.1	0	0.6
Protoplast membrane	III	50	0.1	0	1.0
Insoluble residue	III	47	0.1	0	1.0

protoplast membranes when tested in capillary precipitin tubes. In addition, when compared by immunodiffusion these cell wall extracts formed lines of identity with soluble antigens extracted from membranes. It is clear that neither extensive washing nor differential centrifugation eliminated membrane "contamination" of cell walls.

Membrane, the Antigenic Material in Streptococcal Cell Wall Preparations which Absorbs Heart-Staining Antibody.—These findings suggested that the absorption of heart-staining antibody by streptococcal cell walls might be explained by the persistence of contaminating membrane antigens. As physical methods did not provide cell walls free of membranes, experiments employing a cell wall dissolving enzyme were performed to examine the significance of this membrane contamination in the absorption of heart-staining antibody. The insoluble residues obtained by enzymatic lysis of a number of cell wall preparations were studied both chemically and serologically.

In a typical experiment, a weighed amount of a cell wall preparation of Group A strepto-

cocci was suspended in 0.05 M phosphate-buffered saline (pH 6.5), and thioglycolate-activated Group C phage-associated lysin was added. After incubation at 37°C for 1 hr, the mixture was centrifuged at 20,000 *g* for 45 min. The insoluble residue was collected, washed twice with buffer, twice with water, and lyophilized. The soluble material was dialyzed at 4°C against several changes of water for 48 hr, concentrated by flash-evaporation, and lyophilized.

For comparison, a preparation of purified protoplast membranes received identical treatment.

TABLE VI

Absorption of Immunofluorescence by Fractions Obtained by Enzymatic Lysis of Streptococcal Cell Walls

Absorbed with	Weight	Intensity of staining*		
		Lot I	Lot II	Lot III
	mg			
Cell walls	1	3+	3+	
	2	2+	2+	
	4	+	+/2+	
	6	±	±/+	
Insoluble residue of cell walls	1	±	±/+	
	2	0	0	
	4	0	0	
	6	0	0	
Soluble material of cell walls	1	3+	3+	
	2	3+	3+	
	4	3+	3+	
	6	3+	2+/3+	
Protoplast membranes	1			±/+
	2			0
Insoluble residue of protoplast membranes	1			±/+
	2			0
Unabsorbed globulin control		3+	3+	3+

* Complete absorption read as 0.

Treatment of the cell walls with phage-associated lysin, a muramidase which splits bonds in the mucopeptide of the cell wall, led to solution of more than 75% of the wall. The soluble material contained 96% of the cell wall sugars, rhamnose and glucosamine, while no soluble material was released following treatment of the protoplast membranes with the lysin. On the other hand, as shown in Table V, the insoluble residues that remained after solution of the cell walls had chemical compositions similar to that of the protoplast membrane and quite differ-

ent from those of the original cell wall preparations. Both the residue and the membrane were free of rhamnose and glucosamine, and both contained larger amounts of glucose.

Table VI records the results of absorption studies which compared the relative effectiveness of equal weights of the original cell wall preparations, the two fractions of enzyme-treated walls, and protoplast membranes in removing the heart-staining antibody from streptococcal antisera. The data show that as little as 1 mg of either the insoluble residue or the protoplast membrane essentially abolished all immunofluorescent reactivity, and was as effective as 6 mg of the cell wall preparations.

The insoluble residue representing only one-fifth of the weight of the cell wall preparation was more than five times as active in quenching fluorescence. In addition to its chemical similarity to the protoplast membrane, soluble extracts of this residue formed bands of identity with extracts of membranes when both were tested with membrane antiserum in a double diffusion system. Since the soluble material did not contain membrane antigens and did not absorb any heart-reactive antibody, it was evident that the absorptive capacity of the cell wall was due to that portion of the cell membrane which remained associated with the wall during the isolation process.

Soluble Heart-Reactive Antigens from the Streptococcal Membrane.—The establishment of the membrane of the Group A streptococcus as the locus of the heart-related antigen led to attempts to isolate the specific antigen involved in this reaction. Soluble antigen preparations suitable for precipitin studies had been previously obtained by treating protoplast membranes with various proteolytic enzymes or with hot HCl at pH 2 (13). Extracts of membranes were prepared according to these procedures. Additional extracts were obtained by the hydrolysis of membranes with pepsin at pH 2 for 2 hr. After neutralization and centrifugation, the presence of heart-reactive antigenic material in each supernatant was determined by its effectiveness in absorbing the heart-staining antibody. It was found that extracts prepared either with pepsin or with hot HCl removed the heart-staining antibody from streptococcal sera, while those prepared with trypsin or chymotrypsin had no effect.

The portion of the HCl extracted antigen which absorbed the heart-staining antibody appeared to be primarily a protein moiety. This was suggested by the fact that rapid destruction of the heart-reactive antigen occurred following digestion with enzymes such as trypsin, and by the fact that defatting the membrane by the method of Folch (20) prior to HCl extraction did not affect the activity of the extract. Further details about the immunochemical nature of the heart-reactive antigen of the Group A streptococcal membrane will be reported later.

A Sarcolemmal Fraction from Cardiac Muscle which Reacts with Streptococcal Antibody.—As the sarcolemma seemed to be the part of the cardiac muscle cell

which stained with the streptococcal antibody, an investigation of this structure was begun. A fraction, isolated from human heart muscle by modification of a method used to obtain the sarcolemma of skeletal muscle (21), was found by phase-contrast microscopy to consist largely of flattened cross-striated sheathlike structures. When these sheaths were examined by electron microscopy, the cross-striation was still present, but cytoplasmic structures were not apparent. In addition, these "sarcolemmal sheaths" appeared chemically similar to the sarcolemma of skeletal muscle (22). They contained less than 1% nucleic acid, 70% protein, and 15% lipid.

The cross-reaction between streptococci and heart tissue was supported by the fact that as little as 1 mg of these sarcolemmal sheaths absorbed the heart-staining antibody from streptococcal antisera. HCl extracts of sarcolemma also were active in blocking this reaction. Treatment of the sarcolemmal sheaths with collagenase or defatting did not alter the ability of the residues to absorb the heart-staining antibody. Furthermore, antisera prepared by Rothbard and Watson (23) to various mammalian collagens did not stain the heart sections, and antisera to streptococcal membranes used in this study did not stain collagen in the immunofluorescent test system employed by Rothbard and Watson.

These experiments suggested that neither collagen nor lipid was involved in the serological reaction between streptococci and sarcolemma since the streptococcal related antigen remained after removal of these components. It is likely that the sarcolemmal residue still contained the so called amorphous fraction, considered to be in part a mucopolysaccharide complex, and immunochemical studies of this fraction of the sarcolemma are in progress. Although the experimental evidence is preliminary, it seems possible that the immunological cross-reaction described in this report involves a "membrane structure" of cardiac muscle as well as the cell membrane of Group A streptococci.

DISCUSSION

The existence of an immunological relationship between the hemolytic streptococcus and mammalian tissue is now well established. In the studies reported here, antisera to Group A and A variant streptococci from a wide variety of serological types were shown to contain an antibody which reacted with heart muscle. Not only did the cardiac myofibers stain with the fluorescein-labeled streptococcal antibody, but smooth muscle present in the endocardium and in the media of arterioles also reacted. Although intense staining was observed in the region of the sarcolemma, exact localization of the antibody-binding site within the muscle cell was not possible.

Heart-reactive antibody was found in most Group A and A variant and some Group C antisera, but was not present in sera to other streptococcal groups and other Gram-positive cocci. Antisera to streptococcal membranes produced extremely bright staining of cardiac tissue; an indication that the cell membrane

was the site of the antigenic determinant of the heart-reactive antibody. These findings coupled with the following experimental evidence clearly showed that this antigen was indeed a unique property of the cell membrane of the Group A streptococcus.

First, antisera to purified membranes of Group A streptococci, free of cell wall and cytoplasmic constituents, had high titers of heart-reactive antibody. In contrast, antisera to membranes from other streptococcal groups and other Gram-positive cocci did not react with heart tissue. Second, this immunofluorescent staining reaction was extinguished by absorbing the antisera either with small amounts of purified Group A streptococcal membranes or with soluble antigenic extracts of these membranes. Third, not only do streptococci of Groups A, C, and G have a number of biological characteristics in common, but their membranes are immunologically related to each other (13). Thus, the detection of heart-reactive antibody in a number of Group C antisera was not surprising, and its absorption from Group A sera by membranes of Groups C or G lend further credence to the localization of the antigen within the cell membrane.

Finally, antisera absorbed with cytoplasmic material still stained brightly, and although absorption with cell walls of Group A streptococci did appear to inhibit immunofluorescent staining, even the purest of cell wall preparations were shown to contain large amounts of cell membrane. After the cell wall preparation was dissolved with a muralytic enzyme, the insoluble residue, chemically and serologically very similar to the cell membrane, absorbed the heart-staining antibody while the soluble portion of the cell wall did not. This experiment was the final link in the chain of evidence which pointed to the streptococcal membrane as the locus of this heart-reactive antigen.

These findings stand in contrast to those that have been reported by Kaplan. He described a cross-reactive antigen present in the cell walls of a limited number of Group A streptococcal strains, and noted that this antigen was a protein with physical and chemical properties which appeared similar to but distinct from those described for the type-specific M protein. Our results showed that all Group A streptococci possessed a heart-reactive antigen, and that this antigen was a part of the cell membrane. Furthermore, while the sensitivity of this antigen to a number of proteolytic enzymes suggested its protein nature, its presence in streptococcal strains containing little or no M protein served to confirm that it was not related to this type-specific protein.

Differences between the immunological reaction described by Kaplan and that reported here appear significant, but may simply reflect the difficulties encountered in separating and purifying bacterial cell walls. Similarities such as the appearance and distribution of the immunofluorescent staining produced by both antibody systems coupled with the unlikely possibility that two different

heart-reactive antigens exist, suggest that we are studying the same immunological system.

The recognition that rabbit antisera to Group A streptococci contained an antibody capable of binding to mammalian muscle cells led to a search for similar antibodies in the serum of patients with uncomplicated streptococcal infections and with rheumatic fever. Studies to be reported have confirmed the presence of similar heart-reactive antibodies in the serum of such patients. These human sera produced immunofluorescent staining patterns indistinguishable from those that occurred with antisera to protoplast membranes, and this heart-staining antibody in these human sera also was absorbed either with streptococcal membranes or with the sarcolemma of human cardiac muscle.

Neither the significance of this immunological relationship between Group A streptococci and mammalian muscle cells nor the exact nature of the specific antigens is known. However, the appearance of a heart-reactive antibody in response to a streptococcal infection is obviously of great interest. Although the information about the nature of this antibody and its possible role in the pathogenesis of rheumatic fever is fragmentary, the discovery that a bacterial cellular membrane is immunologically related to a cell membrane structure of mammalian tissue may prove to be of broad biological significance.

SUMMARY

By means of the immunofluorescent staining technique, antisera to a wide variety of serological types of Group A and A variant streptococci were found to contain an antibody which reacted with mammalian striated muscle, both skeletal and cardiac, as well as with smooth muscle in the endocardium and in the media of arterioles. Similar heart-reactive antibodies were not present in antisera to most other groups of hemolytic streptococci and to other Gram-positive cocci. Chemical and serological studies clearly pointed to the cell (protoplast) membrane of the Group A streptococcus as the locus of the antigenic determinant of this heart-reactive antibody. In addition, preliminary studies suggested that the reaction between this streptococcal antibody and cardiac tissue represented an immunological relationship between the sarcolemma, the membrane of a mammalian muscle cell and the cell membrane of a bacterium, the hemolytic streptococcus.

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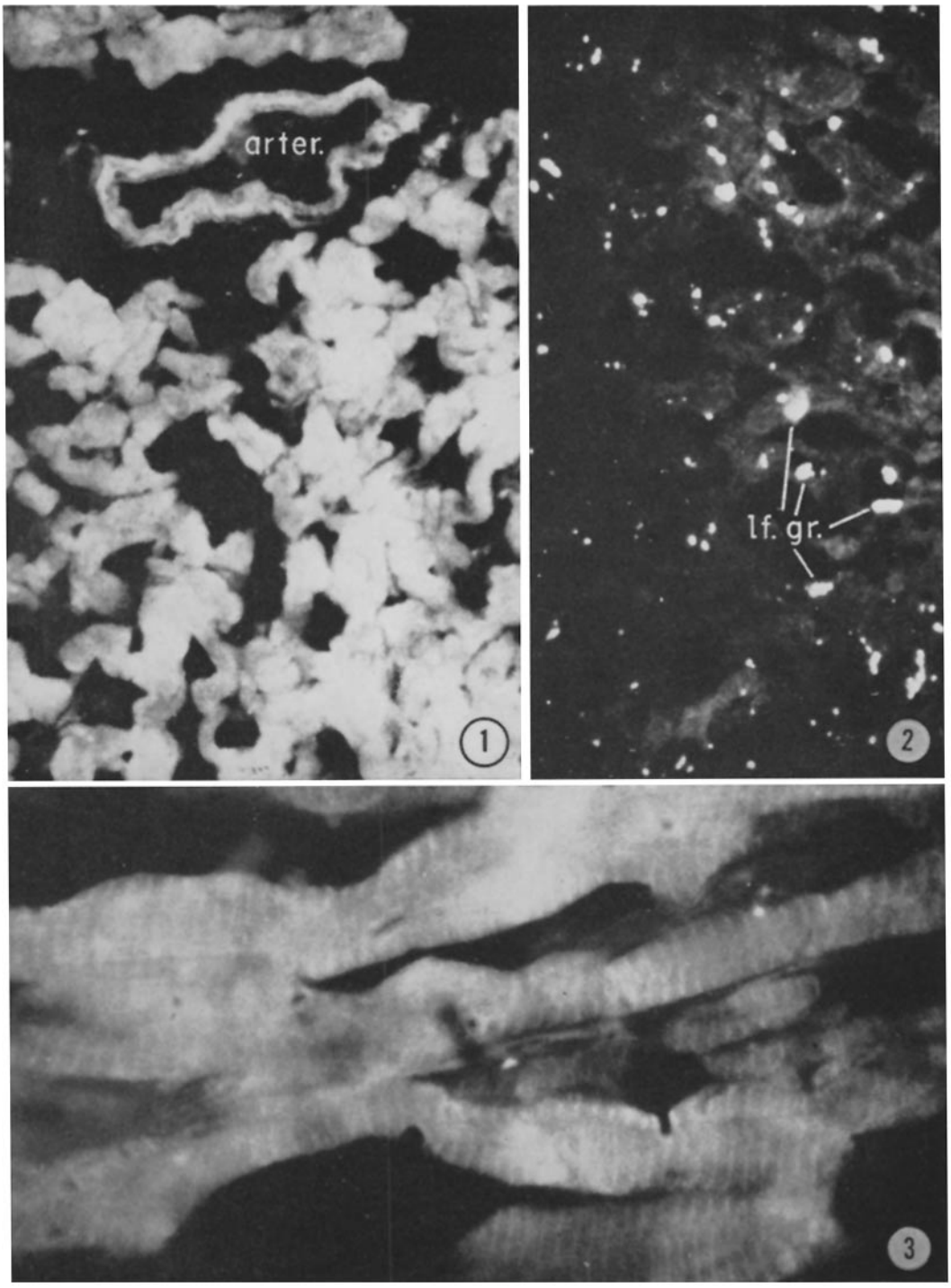
EXPLANATION OF PLATES

PLATE 75

FIG. 1. Immunofluorescent staining of cardiac tissue showing diffuse sarcoplasmic staining. In addition, a small arteriole (*arter.*) is also stained. This section was incubated first with unlabeled rabbit antiserum to membranes of Group A streptococci, and then with fluorescein-tagged sheep anti-rabbit globulin. The specimen was a left auricular appendage from a patient undergoing surgery for mitral valvular disease. The black open spaces are artifacts due to sectioning. × 346.

FIG. 2. The absence of specific fluorescence when another section from the heart specimen shown in Fig. 1 was incubated with antiserum that did not contain heart-reactive antibody. The bright granular areas (*lf. gr.*) represent lipofuschin granules. × 346.

FIG. 3. Cross-striational staining pattern in a longitudinal section of cardiac muscle from a left auricular biopsy specimen of an individual with rheumatic heart disease. Fluorescein-labeled antimembrane globulin was used. × 864.



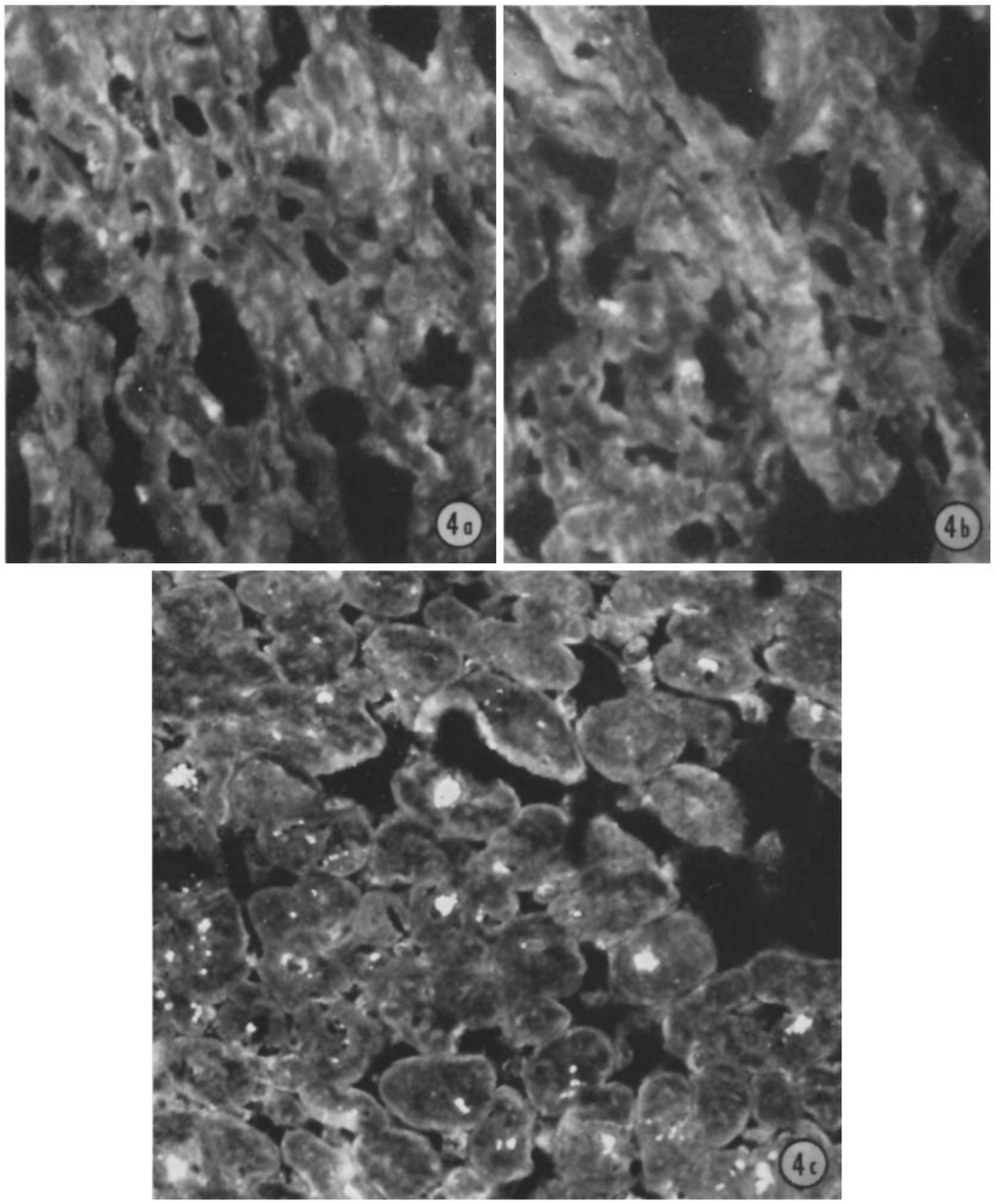
(Zabriskie and Freimer: Group A streptococcus and mammalian muscle)

PLATE 76

FIGS. 4 *a* to 4 *c*. Demonstration of sarcolemmal staining by antisera to membranes of Group A streptococci using the indirect staining technique. × 1250.

FIGS. 4 *a* and 4 *b*. Longitudinal sections of muscle cells from the left ventricle of a 4-yr-old child who died of leukemia.

FIG. 4 *c*. Cross-section of muscle cells from the left auricle of another patient with rheumatic heart disease.



(Zabriskie and Freimer: Group A streptococcus and mammalian muscle)

PLATE 77

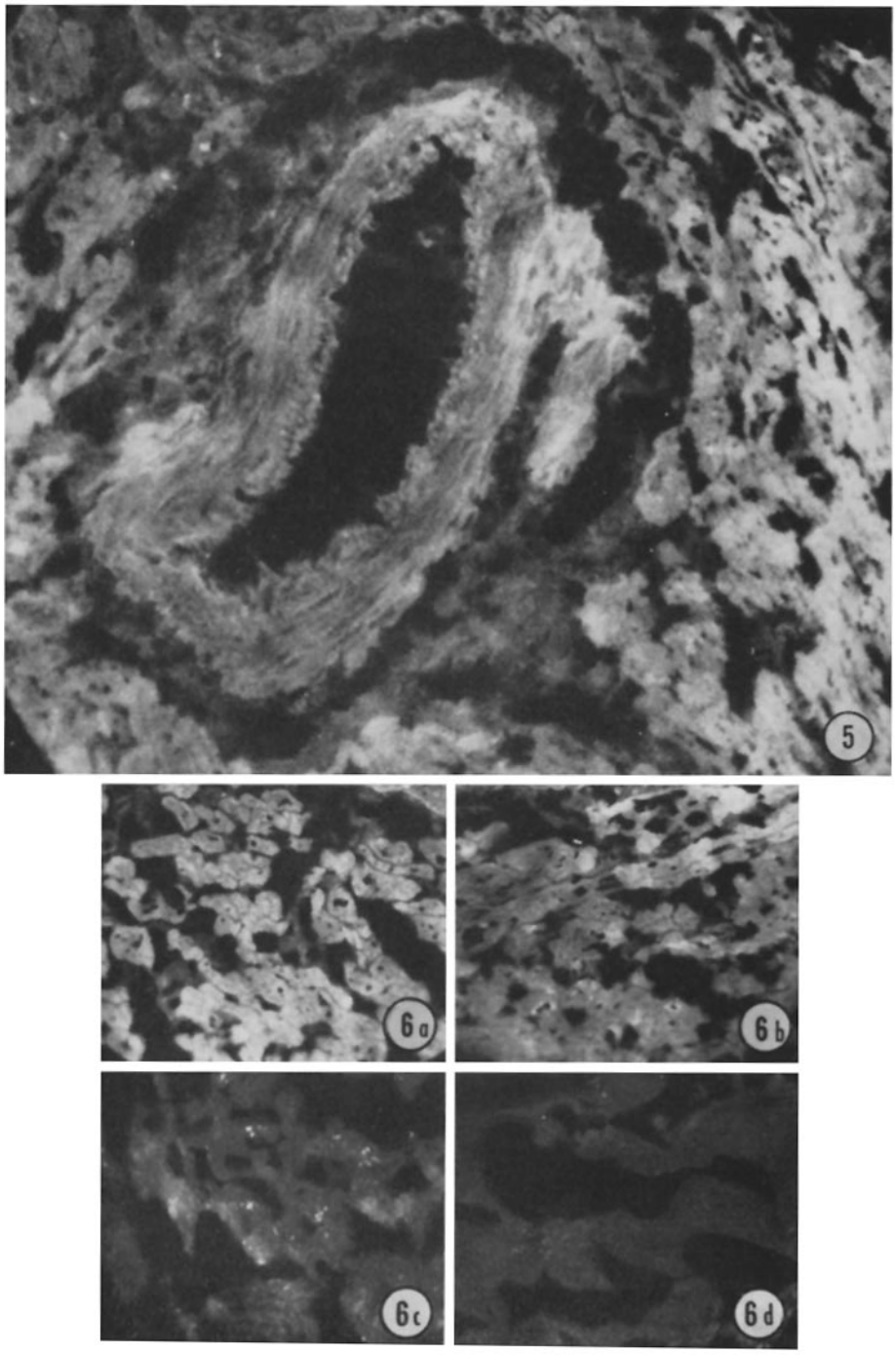
FIG. 5. Direct immunofluorescent staining of the smooth muscle layer of an arteriole as well as the striated muscle in a section from a biopsy of the left auricle of a non-rheumatic human heart. The fluorescein-labeled globulin was prepared against protoplast membranes of Group A streptococci. × 250.

FIGS. 6 *a* to 6 *d*. Quantitative absorption of heart-reactive antibody by increasing weights of antigen. This fluorescein-labeled globulin was prepared against whole Group A streptococci. The sections of heart muscle were from the left auricle of another patient with rheumatic heart disease. Each section was photographed under identical conditions. × 200.

FIG. 6 *a*. Shows the bright (4+) staining produced by the unabsorbed globulin.

FIGS. 6 *b* and 6 *c*. Show progressive removal of heart-staining antibody.

FIG. 6 *d*. Shows the absence of staining (0) after this antibody had been completely removed.



(Zabriskie and Freimer: Group A streptococcus and mammalian muscle)