RELATION OF RHEUMATIC-LIKE CARDIAC LESIONS OF THE MOUSE TO LOCALIZATION OF GROUP A STREPTOCOCCAL CELL WALLS*

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It has been demonstrated that a single intradermal injection of cell wall fragments of Group A streptococci into rabbit skin will initiate a chronic granulomatous lesion characterized by remissions and exacerbations (1–3). A basic feature of the pathogenesis of this experimental lesion is the relationship between lesion activity and the ability of the host to eliminate the cell wall material from the tissue (4). Although the evidence accumulated thus far indicates that direct toxicity of the persisting cell wall structure is of primary importance (5, 6), we are continuing to investigate the role of the immune response of the host in our studies of the mechanism of this lesion.

More recently this experimental model of tissue damage has been extended to the rabbit joint (7) and the mouse heart (8). The available data indicate that all of these models involve a common pathogenic mechanism. This paper extends the histological description of the cardiac lesions produced with crude extract and demonstrates that these lesions can be produced with isolated cell wall fragments. Employing fluorescein or ^{125}I-labeled antibodies specific for Group A streptococcal cell wall antigens, it is readily demonstrable that cell wall material accumulates in the mediastinal lymph nodes and adjacent loose connective tissue and can persist at these sites for at least 10 wk. Furthermore, the cell wall fragments are shown to be localized around the sites of active lesions in the heart.

Materials and Methods

Animals.—An outbred line of Swiss-Webster white mice (Blue Spruce Farm, Altamont, N. Y.) and an inbred C$_3$H strain were used. All animals were male.

Bacterial Fractions.—Further details have been presented previously (2). Group A, Type 3, strain D-58 streptococci were cultured for 16 hr in 30 liters of trypticase-soy broth. The cells were collected in a Servall continuous flow rotor, washed three times with 1 liter of

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cold saline and resuspended to approximately a 300-fold concentration in pH 7.0 M/15 phosphate-buffered saline (PBS). These suspensions were then disrupted in 30 ml aliquots with 30 g of No. 12 glass beads in a Braun MSK shaker with flowing CO_2, using 3 min treatments. The glass beads were allowed to settle and the supernatant removed. The beads were rinsed several times and the rinses pooled with the first supernatant.

Cell Walls.—The suspension of disrupted cells was diluted to approximately 800 ml to reduce viscosity, and centrifuged at 10,000 *g* in a Servall SS-34 rotor for 30 min. The top layer of sediment was carefully resuspended in buffered saline and this cycle of centrifugation was repeated three times. The final suspension was treated with ribonuclease, trypsin, and Duponol as previously described (2). The Duponol was removed by washing the cell walls five times with water at 25°C. The cell walls were then dialyzed against distilled water and lyophilized. For injection, 100 mg was suspended in 15 ml of PBS and sonicated within a glass vial for 2 min in the cup of a 9KC Raytheon sonic oscillator, which contained 5 ml of water.

Cell Wall Fragments.—100 mg of the cell wall preparation was suspended in 15 ml of PBS and treated for 75 min at maximum amplitude in the cup of the Raytheon 9 KC sonic oscillator. This yielded an opalescent stable suspension. This preparation was very polydisperse, and the range of particle size has not been determined. With comparable preparations it has been demonstrated that most of the toxicity, as defined by nodular lesions in rabbit skin, is sedimented at 90,000 *g* but not 27,000 *g*.

Soluble Fraction of Streptococcal Cells.—The first supernatant obtained after centrifugation of the crude extract at 10,000 *g* was further centrifuged twice at 37,000 *g* for 1 hr in a Servall SS-34 rotor. The supernatant was dialyzed against deionized water at 4°C and lyophilized. This contains the soluble cytoplasmic components and only trace amounts of cell wall can be detected by rhamnose determinations and immunodiffusion. This will, of course, depend a great deal upon the degree of solubilization of the cell wall during the disruption of the cells. For injection, 100 mg was suspended in 15 ml of PBS and a clear solution obtained by sonication for 2 min as described for the cell wall material.

Crude Sonic Extracts.—Washed cells from either Group A or Group D streptococcal broth cultures were resuspended in PBS and disrupted with a Bronson sonifier, without glass beads, employing 60 min treatment. The crude extract was filtered through Millipore 3 μ, 1.2 μ, and 0.45 μ filters in succession. After the last filtration, a portion of the filtrate was tested for sterility by plating on a sheep blood agar plate. Rhamnose determinations were done on each lot of sonicate as an estimate of the cell wall material present and/or a method of standardization of dosage.

Preparation for Injection of Mice.—For experiments comparing cell wall and soluble fractions each preparation was diluted to 20 ml with pH 7.0 PBS and held at 62°C for 30 min prior to injection. Sterility was checked by plating 0.1 ml samples on sheep blood agar plates.

Preparation of Tissue for Histological Examination.—Tissues were fixed in 10% neutral formalin and the paraffin-embedded sections were stained with hematoxylin and eosin or Giemsa.

Identification of Group A Streptococcal Cell Wall Antigens in Tissue.—The preparation of antibodies specific for the polysaccharide or mucopeptide cell wall antigens, their labeling with fluorescein isothiocyanate or ^{125}I, and the fluorescence microscopy or radioautography techniques have been described in detail (4, 9).

The tissues from all Swiss-Webster mice, injected intraperitoneally or intravenously with crude sonic extract, were fixed in formalin, paraffin-embedded, and sections 4 μ thick prepared. These could only be studied by radioautography with the radioantibodies because of the autofluorescence of mouse tissues prepared in this manner.

Tissues from the C_3H strain of mice, injected with isolated cell wall fragments, were fixed in cold ethanol and processed by the method of Sainte-Marie (10). These tissues were examined both by fluorescein-labeled and ^{125}I-labeled antibodies.

Controls consisted of tissues stained with normal labeled globulin or blocking with unlabeled antibody prior to the application of labeled antibody. Other controls consisted of tissue from mice injected with buffered saline or with Group D streptococcus cell wall preparations. The latter is an important control for nonspecific sticking of globulin in areas of inflammation.

RESULTS

Production of Cardiac Lesions with Cell Wall Fragments.—

Swiss-Webster mice weighing 24–28 g were divided into four groups of 10 mice. Each group was injected intraperitoneally with one of the following preparations: (*a*) 10 mg of Group A streptococcal cell walls sonicated for 2 min to give an even suspension, (*b*) 10 mg of fragments of the same cell wall preparation produced by sonication for 75 min, (*c*) 10 mg of the soluble, cytoplasmic fraction of the cells, (*d*) 2 ml of the phosphate-buffered saline, pH 7.0, in which the above preparations were suspended. Two mice from each group were killed at weekly intervals for 5 wk after injection and the tissues prepared for histological examination.

All mice injected with the cell wall fragments developed a carditis with the essential features described previously (8), although the lesions are not as extensive. None of the mice injected with the other preparations, including nonfragmented cell walls, showed heart lesions. These results are tabulated in Table I. The histopathology in these animals was compared with lesions produced with crude extract containing a similar amount of cell wall material (3.5 mg of rhamnose). The characteristic features are summarized as follows: The carditis was characterized by involvement of the pericardium, myocardium, coronary arteries, and the heart valves. The lesions of the pericardium consisted of focal collections of mononuclear cells and neutrophils on the epicardial surface and infiltration of the subepicardial tissue by similar cells. The myocardial lesions were characterized by areas of necrosis which were surrounded by a variety of cells including mononuclear and giant cells with irregular basophilic cytoplasm, indistinct cell borders, and nuclei typical of Anitschkow's myocytes. These lesions were most numerous in the wall of the left ventricle, intraventricular septum, and papillary muscles of the left ventricle. Many of the focal myocardial lesions were located adjacent to coronary arteries and some appeared to surround arteries (Figs. 1 and 2). Changes in the coronary arteries were observed throughout the experiments. The type of lesion observed most commonly is illustrated in Fig. 3. Changes consisted of endothelial hyperplasia, edema of the media, and accumulations of mononuclear cells in the media and adventitia. Arterial necrosis and thrombosis were not observed. The cusps and bases of the aortic and mitral valves were involved by the inflammatory process. There was edema of the interstitial tissue with accumulation of neutrophils and mononuclear cells including Anitschkow's myocytes.

The roots of the mitral and aortic valves and adjacent myocardium showed areas of necrosis and accumulations of a variety of inflammatory cells similar to those seen in the focal lesions of the myocardium described above. The endocardium adjacent to the valves showed hyperplasia and infiltration with inflammatory cells. In addition to the diffuse inflammatory process of the valves, nodular lesions were observed in which the endocardium was elevated by dense focal accumulations of inflammatory cells. Inflammation of a mitral valve is illustrated in Fig. 4.

Localization of Cell Wall Antigens in Relation to Cardiac Lesions.—As described above, the lesion process in mice injected with either crude extract of sonic disrupted Group A streptococci or isolated cell wall fragments was essentially similar. Likewise, the distribution and persistence of cell wall antigens were comparable.

TABLE I

Comparison of the Cardiotoxic Properties of Cellular Components of Group A Streptococci

	Amount injected	No. of mice with carditis per total No. injected
Cell walls, *mg*	10	0/10
Cell wall fragments, *mg*	10	8/8
Soluble fraction, *mg*	10	0/10
Buffer, *ml*	2	0/10

A total of 25 mice from a series involving the intravenous or intraperitoneal injection of Swiss-Webster animals with the crude sonic extract, and 14 mice from a group of C_3H animals injected intraperitoneally with isolated Group A cell wall fragments, were examined for localization of cell wall antigens. Animals were killed at intervals from 1 day to 10 wk after injection of either Group A streptococcal cell wall fragments or crude extract, Group D streptococcal cell wall fragments or crude extract, or buffered saline. Usually two or three animals from each group were studied at the selected time intervals. Tissue sections were examined by fluorescence microscopy or radioautography using labeled antibodies specific for Group A polysaccharide, or mucopeptide. The controls used are described under Materials and Methods.

Cell wall antigens could be readily demonstrated in the spleen, liver, and mediastinal lymph nodes in samples obtained at every interval from 24 hr to 10 wk. The localization of antigen in the lung was more variable as judged by intensity of silver grains, while antigen was seen only occasionally in glomeruli of the kidney and not in the thymus.

Antigen was detected at 2–4 days in the pericardium, with some variability between animals, and continued to be present in most animals through 5

wk. Cell wall antigen was also observed, with individual variation, in the myocardium, atrium, and mitral valve.

With regard to the relationship of cell wall material to the histopathology, we have concluded that an active lesion process is associated with the presence of antigen. With the greater precision of the fluorescent antibody technique, the antigens were always observed at the periphery of the sites of granulomatous inflammation, as illustrated in Figs. 5–8. On the other hand, antigen was frequently found in the heart in the absence of any obvious tissue damage (Figs. 9–12).

It is interesting that antigen was always found in the mediastinal lymph nodes and appeared as a continuum through the loose connective tissue around the blood vessels in this area. Fig. 13 illustrates the intensity of the fluorescence in the mediastinal node 8 days after injection. The intensity of the radioautograph of the loose connective tissue 10 wk after injection is recorded in Fig. 14.

DISCUSSION

The lesions produced in the hearts of mice injected with Group A streptococcal cell wall fragments are strikingly similar to the cardiac lesions of rheumatic fever. The studies reported here indicate that the active lesion process is associated with the localization of the cell wall material around the site of tissue damage. Other studies conducted in rabbit skin have demonstrated the toxicity of Group A streptococcal cell walls, probably functioning through a direct toxic activity of the mucopeptide moiety (11). The mechanism by which the cardiac lesion could develop as a result of deposition of cell walls is not known. It could conceivably involve hypersensitivity or an autoimmune reaction. Although our studies with this material in the other tissue models do not support this concept, we are pursuing the possibility that the immune response of the host is directly involved in the pathological events. Serological studies show that mice injected with the dose of cell walls employed here do develop cell wall agglutinating antibodies 4–7 days after injection, and then maintain a rather constant level of circulating antibody for at least 24 wk (12).

It was noted that cell wall antigen was found only at the periphery of the sites of granulomatous inflammation, and was frequently seen in the heart in the absence of obvious lesion activity. This suggests that the lesions develop in the process of the host's efforts to eliminate cell wall material, whatever mechanisms this may involve. Thus, cell wall is not detected in advanced lesion processes, and those apparently normal sites where it is found represent areas of potential lesion development. Since the mice are producing antibody, the inability to detect cell walls within an active lesion could be the result of blocking of antigenic sites by the host's antibody. We have not, as yet, been

able to demonstrate mouse immunoglobulins in the lesions. This critical question must be resolved by complementing our studies with internally labeled cell walls. The bound globulin, which Kaplan et al. (13) have demonstrated in hearts from patients dying of acute rheumatic fever, could be interpreted as antibody against localized Group A streptococcal cell wall antigens, rather than the autoantibodies reacting with myofiber sarcolemma which these workers postulate. The fact that the bound globulin in human hearts is not found in the Aschoff lesion but is associated with uninvolved tissue (13) would, in fact, fit with our observations.

The relative ineffectiveness of the large cell wall suspension compared to cell wall fragments is similar to the previous observation on an optimum particle size for development of nodular skin lesions (14). This may reflect the distribution and localization at a critical site, or differential processing and elimination of different size particles.

The accumulation and persistence of cell wall material in the mediastinal lymph nodes is remarkable, and the possibility that lymphoid tissue represents the mode of translocation of Group A streptococcal cell walls to the heart is being investigated.

SUMMARY

Mice injected intraperitoneally with isolated cell wall fragments of Group A streptococci develop a carditis similar to that previously observed in mice injected with crude extracts of this organism. Neither the soluble cytoplasmic components of Group A streptococcal cells nor the nonfragmented cell walls produced carditis in this experimental model. Fluorescein and ^{125}I-labeled antibodies specific for Group A streptococcal cell wall antigens were used to demonstrate that, for 5 wk after injection, cell wall material is localized around the sites of active lesions in the heart. In addition, the cell wall antigen accumulates in the liver, spleen, mediastinal lymph nodes, and the adjacent loose connective tissue, where it persists for at least 10 wk.

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FIG. 1. Myofiber necrosis and accumulation of mononuclear cells. Section from a mouse sacrificed 2 wk after injection of crude extract of Group A streptococcal cells. × 513.

FIG. 2. Granuloma adjacent to a coronary artery. Section from a mouse sacrificed 3 wk after injection of crude extract of Group A streptococcal cells. × 102.

FIG. 3. Arteritis of a coronary artery. Section from a mouse sacrificed 1 wk after injection of crude extract Group A streptococcal cells. × 513.

FIG. 4. Inflammation of the mitral valve. Section from a mouse sacrificed 3 wk after injection of crude extract of Group A streptococcal cells. × 102.

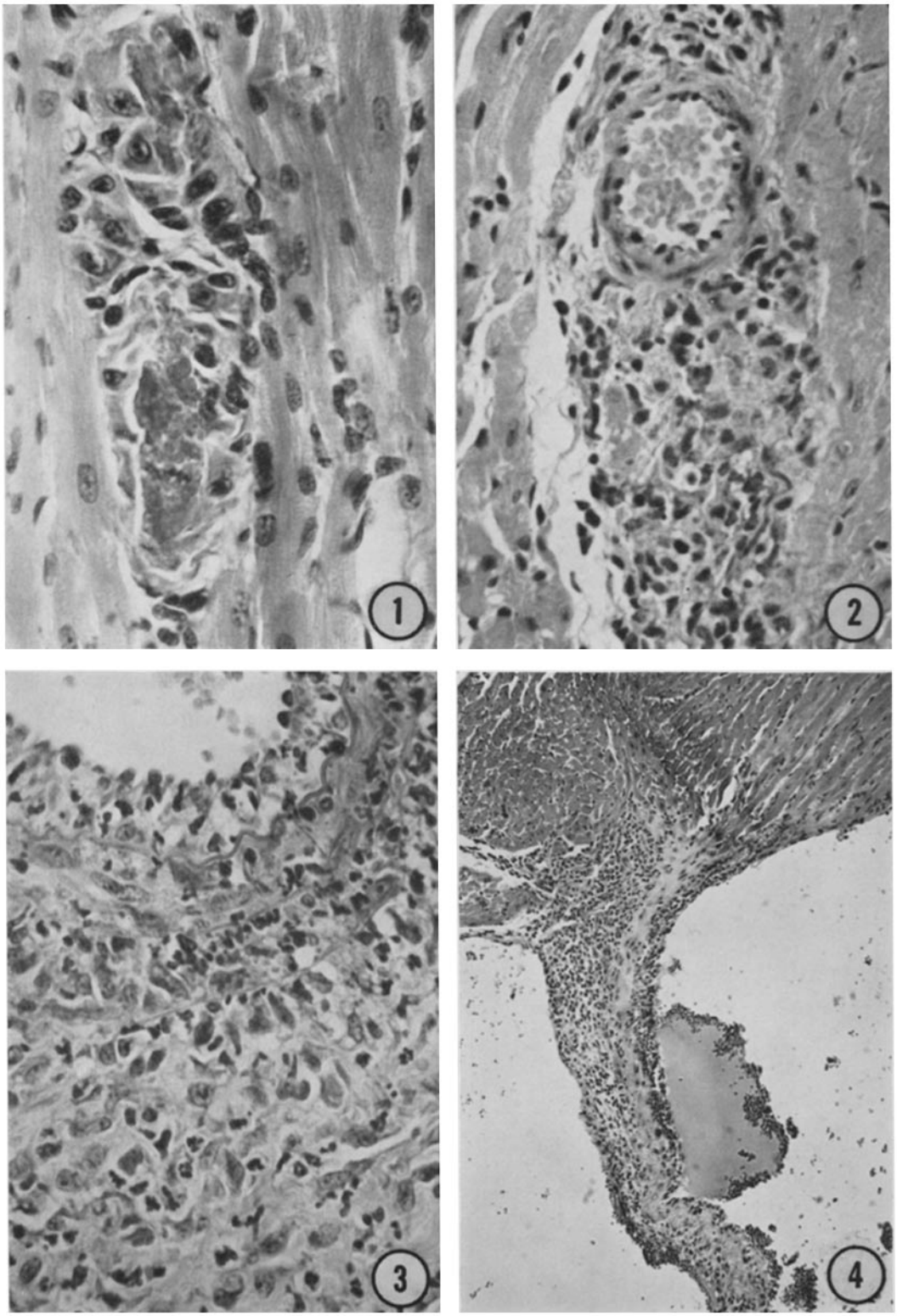
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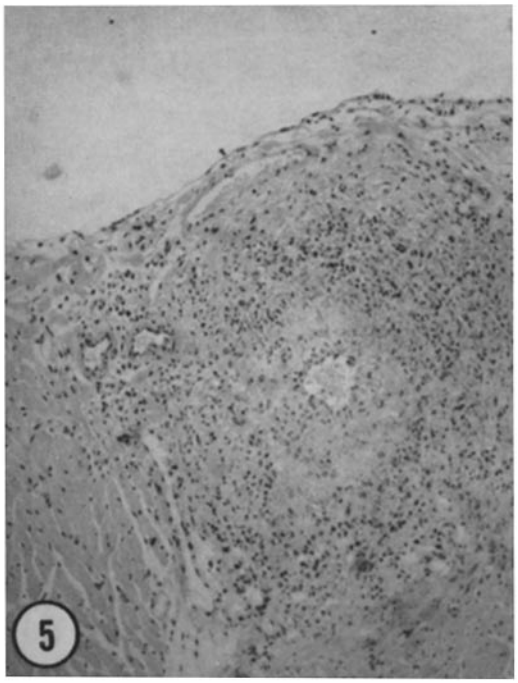
FIG. 5. Granulomatous lesion around coronary artery in a C_3H mouse injected intraperitoneally 2 wk previously with Group A streptococcal cell wall fragments. Giemsa stain. × 82.

FIG. 6. Higher magnification of left middle area of Fig. 5. × 342.

FIG. 7. Same section shown in Fig. 6, stained with fluorescein-labeled anti–Group A polysaccharide. Fluorescence can be identified with specific mononuclear cells. 30 sec exposure. × 342.

FIG. 8. Serial section of heart shown in Figs. 5–7. Stained with fluorescein-labeled normal rabbit globulin. 40 sec exposure. × 342.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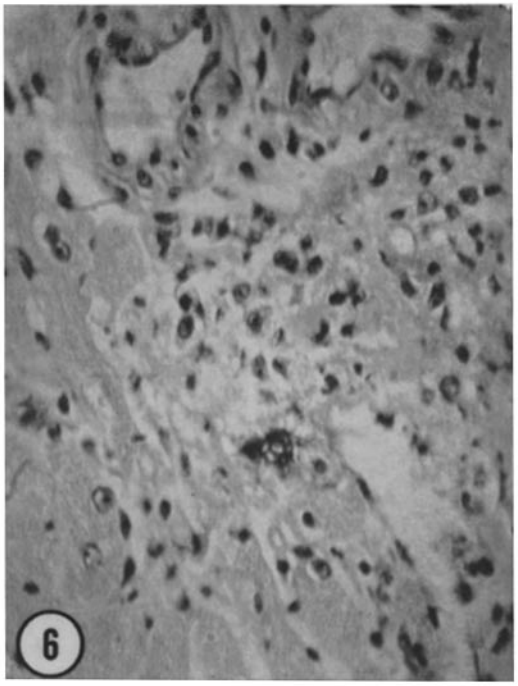
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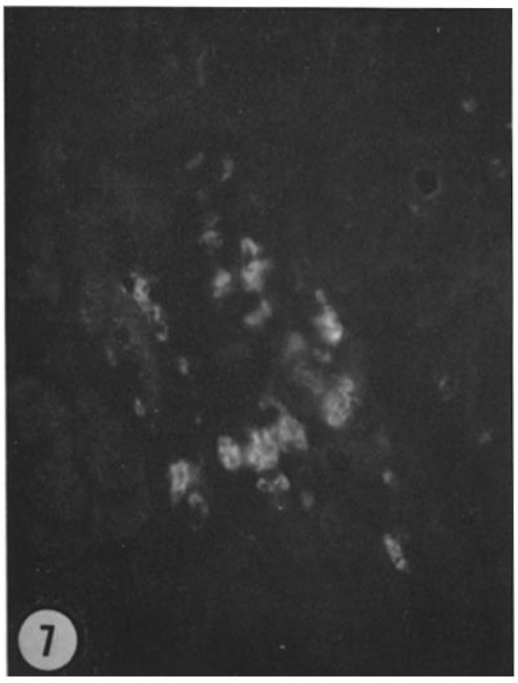
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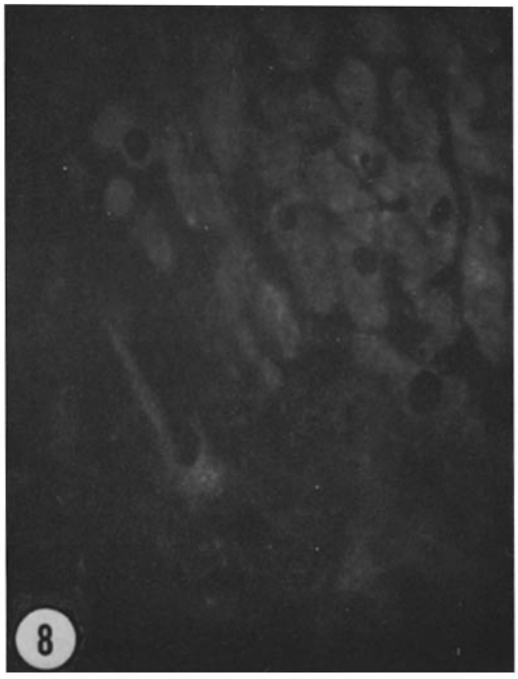
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FIG. 9. Heart taken from mouse injected 2 wk previously with Group A streptococcal cell wall fragments. Minimal inflammation in valve and adjacent tissue. Giemsa stain. × 73.

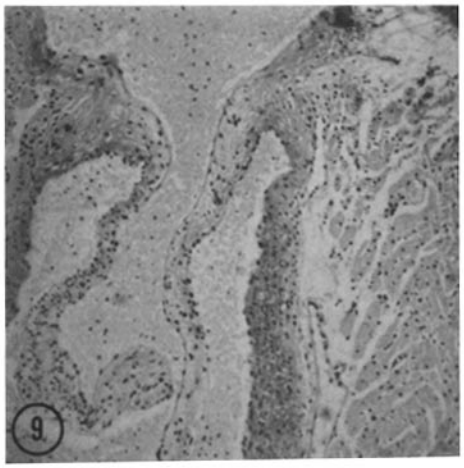
FIG. 10. Serial section of heart shown in Fig. 9. Approximately same area as shown in Figs. 11 and 12. Control showing absence of fluorescence when stained with fluorescein-labeled anti-Group D polysaccharide. 90 sec exposure. × 308.

FIG. 11. Higher magnification of right middle area of Fig. 9. × 308.

FIG. 12. Same section shown in Fig. 11, stained with fluorescein-labeled anti-Group A polysaccharide. Fluorescence can be identified with specific cells seen in Fig. 11. 40 sec exposure. × 308.

FIG. 13. Lymph node adjacent to heart in C_3H mouse injected 8 days previously with Group A cell wall fragments. Stained with fluorescein-labeled anti-Group A polysaccharide. 30 sec exposure. × 73.

FIG. 14. Loose connective tissue around large vessels in mediastinum. C_3H mouse injected intraperitoneally 10 wk previously with Group A cell wall fragments. Radioautograph of section stained with ^{125}I anti-Group A polysaccharide followed by Giemsa stain after photographic development. × 73.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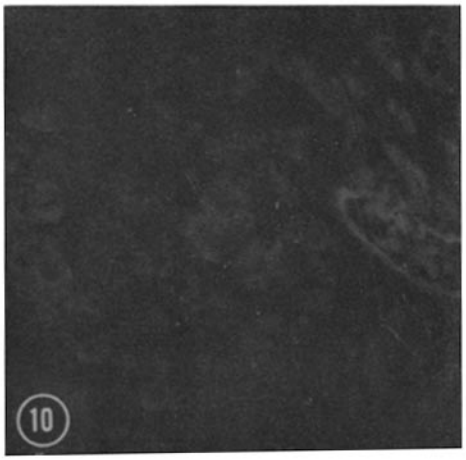
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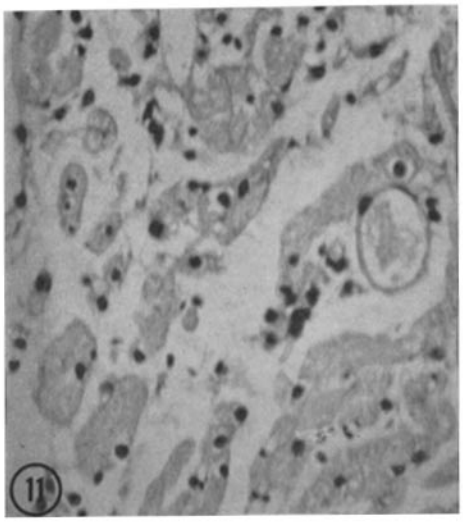
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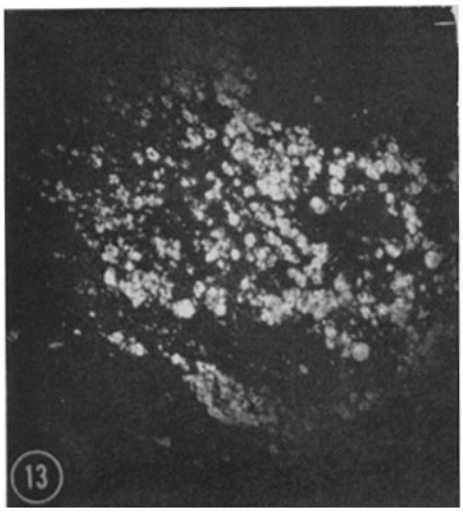
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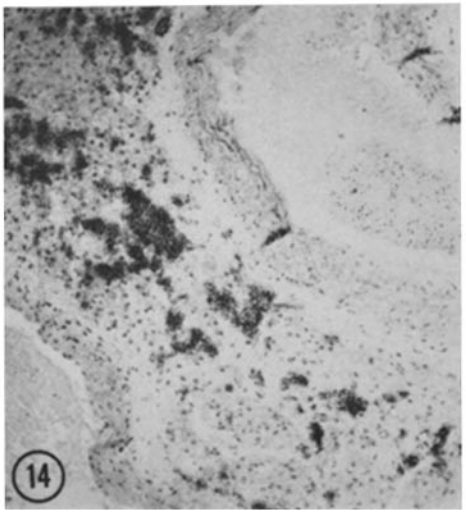
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