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STUDIES ON CELL WALLS OF GROUP A *STREPTOCOCCUS PYOGENES*, TYPE 12

III. INDUCTION OF CARDIAC LESIONS IN MICE BY A HIGHER MOLECULAR WEIGHT FRACTION FROM A DIGEST OF CELL WALLS WITH THE L-11 ENZYME[1]

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SUMMARY A higher molecular weight fraction was obtained by digesting the cell walls of group A streptococci with the *Flavobacterium* L-11 enzyme. On daily intraperitoneal injections into mice (0.5 mg × 4) it induced cardiac lesions similar to those induced by cell wall fragments obtained by sonic oscillation. This shows that for manifestation of cardiotoxicity group A streptococcal cell walls do not require an optimum particle size, but are active as a soluble complex of C-polysaccharide with glycopeptide.

INTRODUCTION

One method to study the pathogenesis of rheumatic fever is to determine whether any cellular components of group A *Streptococcus pyogenes* can induce inflammation of cardiac tissue like that occurring in rheumatic fever. Cromartie and Craddock (1966) showed that cardiac lesions, like those seen in rheumatic fever, were induced in mice by a single intraperitoneal injection of an extract of sonically disrupted group A streptococcal cells. Later, Ohanian, Schwab and Cromartie (1969) induced similar lesions in mice by intraperitoneal injection of isolated cell wall fragments of group A streptococci. Rotta and Bednář (1969) reported that intravenous injection of a peptidoglycan (mucopeptide) obtained from group A streptococcal cell walls by removal of group-specific C-polysaccharide with hot formamide induced focal mononuclear cell infiltration and/or granulomatous changes in the myocardium of rabbits. The above experiments were performed with group A streptococcal cells or cell walls which had been "solubilized" (that is finely disrupted by sonication to yield a stable suspension.). The second paper of this series (Hamada et al., 1971) reported that a higher molecular weight frac-

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tion (HMW) isolated from an enzymatic digest of the cell walls of group A *Streptococcus pyogenes* (type 12, strain S.F. 42) by gel filtration retained the pyrogenicity and some other biological properties of the cell walls.

This paper reports studies on the ability of HMW solution to induce cardiac lesions similar to those produced by cell wall fragments obtained by sonication.

MATERIALS AND METHODS

1. *Animals*

Male, inbred ddO strain mice were obtained from the Breeding Station for Laboratory Animals, Osaka University. They were fed on mouse chow MF (Oriental Yeast Co., Osaka) and water *ad libitum*. Animals were 5 weeks old at the start of experiments, and they weighed 20 to 25 g.

2. *Streptococcal strains*

Group A, type 12, strain S.F. 42, and group A, type 6, strain S43/100 were used for preparation of cell walls. The latter strain was generously supplied by Dr. R. M. Krause of the Rockefeller University.

3. *Preparation of purified cell walls.*

Bacteria were grown in Trypticase Soy Broth (BBL) and cell walls were obtained as previously described (Hamada et al., 1971).

4. *Higher molecular weight fraction (HMW)*

HMW was isolated by gel filtration from a digest of the cell walls of strain S.F. 42 with the L-11 enzyme. It was the same preparation as that used in the previous study. Special care was taken to prevent the cell walls and HMW from being contaminated with extraneous endotoxins (Hamada et al., 1971).

5. *Assay of the ability of cell walls or HMW to induce cardiac lesions in mice*

A purified preparation of cell walls or HMW was suspended or dissolved in physiological saline solution at a concentration of one mg/ml. The cell walls were subjected to sonic vibration in a KUBOTA sonic vibrator (10 kc/sec, KMS-100) for 30 min at maximum amplitude to give an even suspension. The sonicated preparation of cell walls and the HMW solution were sterilized by pouring them into Petri dishes of 9 cm diameter to a depth of about 2 mm, and irradiating them from a distance of 20 cm with a 10 W ultraviolet lamp (GL-5, Tokyo Shibaura Electric Co. Ltd., Tokyo) for 20 min, with occasional shaking.

Seventy mice divided into four groups of 10 or 15 mice. The animals in each group were injected intraperitoneally with one of the following test specimens (in 0.5 ml of physiological saline): Experiment 1. Sonically disrupted cell walls of strain S.F. 42 (2 mg in 4 doses of 0.5 mg, once a week for 4 weeks. 10 mice); Experiment 2. Sonicated cell walls of strain S43/100 (2 mg in 4 doses of 0.5 mg, once a week for 4 weeks. 10 mice); Experiment 3. HMW from strain S.F. 42 (2 mg in daily doses of 0.5 mg for 4 days. 15 mice); Controls. Physiological saline (2.0 ml. 10 mice as controls for experiment 1 or 2, and 15 mice as controls for Experiment 3). Five mice from each group were sacrificed 14 and 28 days (Experiments 1 and 2) or 2, 7 and 28 days (Experiment 3) after the last injection, and their heart, lungs, liver, spleen and kidneys were removed for histological examination.

6. *Histological examination*

Tissues were fixed in 10% neutral formalin and embedded in paraffin. Sections were cut and stained with hematoxylin and eosin. The incidence and extent of lesions were arbitrarily graded as 0 (no lesion), ± (slight lesion), and + (definite lesion).

RESULTS

1. *Production of cardiac lesions with cell wall fragments*

As positive control experiments, ddO mice were injected intraperitoneally with sonically disrupted cell walls of group A *Streptococcus pyogenes*, type 12 (strain S.F. 42) or type 6 (strain S43/100). Tables 1 and 2 show that most of the mice that received the cell wall fragments develop pathological alterations involving the pericardium, myocardium, valves and coronary arteries. The lesions of the pericardium of mice sacrificed 4 weeks after the last injection consisted of a focal accumulation of mononuclear cells and neutrophils in the epicardial surface (Fig. 1). The pericardium

TABLE 1. *Cardiac lesions in mice produced by intraperitoneal injections of sonically disrupted cell wall fragments from group A streptococci (type 12, strain S.F. 42)*

Days after last injection	Mouse No.	Heart				Blood vessels		Pathological findings	
		Peri-cardium	Myo-cardium	Endo-cardium	Heart valves	Coronary arteries	Aorta		
14	1	0	0	0	0	0	0	0	
	2	0	0	0	0	0	0	0	
	3	0	±	0	0	0	0	±	3/5[a]
	4	0	±	0	0	±	0	+	
	5	0	0	0	±	0	0	±	
28	6	+	±	0	0	0	+	+	
	7	0	0	0	0	±	0	±	
	8	0	0	0	±	0	0	±	4/5[a]
	9	0	0	0	0	0	0	0	
	10	±	±	0	0	0	0	+	

Cell wall fragments (0.5 mg each) were injected intraperitoneally once a week for 4 weeks.
0 : *no lesion.* ± : *slight lesion.* + : *definite lesion.*
a No. of positive / No. of test.

TABLE 2. *Cardiac lesions in mice produced by intraperitoneal injections of sonically disrupted cell wall fragments from group A streptococci (type 6, strain S43/100)*

Days after last injection	Mouse No.	Heart				Blood vessels		Pathological findings	
		Peri-cardium	Myo-cardium	Endo-cardium	Heart valves	Coronary arteries	Aorta		
14	1	0	±	0	0	0	0	±	
	2	0	0	0	0	0	0	0	
	3	0	0	0	0	0	0	0	3/5[a]
	4	0	±	0	±	0	0	+	
	5	0	±	0	±	0	0	+	
28	6	+	+	0	±	+	+	+	
	7	0	±	0	±	0	0	+	
	8	+	+	0	0	0	0	+	4/5[a]
	9	0	±	0	0	0	0	±	
	10	0	0	0	0	0	0	0	

Cell wall fragments (0.5 mg each) were injected intraperitoneally once a week for 4 weeks.
0 : *no lesion.* ± : *slight lesion.* + : *definite lesion.*
a No. of positive / No. of test.

TABLE 3. *Cardiac lesions in mice produced by intraperitoneal injections of HMW isolated from group A streptococcal cell walls (type 12, strain S.F. 42)*

Days after last injection	Mouse No.	Heart				Blood vessels		Pathological findings	
		Peri-cardium	Myo-cardium	Endo-cardium	Heart valves	Coronary arteries	Aorta		
2	1	0	0	0	0	0	0	0	
	2	0	+	0	0	0	0	+	
	3	+	0	0	+	0	0	+	3/5[a]
	4	+	0	0	0	0	0	+	
	5	0	0	0	0	0	0	0	
7	6	0	0	0	0	0	0	0	
	7	0	+	0	0	0	0	+	
	8	0	0	0	0	0	0	0	3/5[a]
	9	0	0	+	+	0	0	+	
	10	+	0	0	+	0	0	+	
28	11	0	0	+	0	0	0	+	
	12	0	+	0	0	0	0	+	
	13	0	0	0	+	0	0	+	4/5[a]
	14	0	+	0	0	0	0	+	
	15	0	0	0	0	0	0	0	

Test specimens (0.5 mg each) were daily injected intraperitoneally for 4 days.
0 : *no lesion.* + : *definite lesion.*
a No. of positive / No. of test.

of the hearts of animals of this group examined 2 weeks after the last injection showed no lesions. Lesions of the myocardium were characterized by degenerative changes of myofibers and focal accumulation of mononuclear cells. These lesions were observed in the hearts of animals sacrificed 2 and 4 weeks after the last injection. Focal granulomatous lesions were not seen in myocardium of hearts examined 2 weeks after the last injection, but were seen 4 weeks after the last injection (Fig. 2). Heart valves examined 2 and 4 weeks after the last injection revealed slight edema of interstitial tissue with focal accumulation of mononuclear cells and neutrophils (Fig. 3). No significant lesion of the endocardium was seen. Changes in the coronary arteries were not significant, but the proximal portion of the aorta showed lesions characterized by proliferative and exudative reactions involving the intima, media and adventitia (Fig. 4).

Tables 1 and 2 show that pathologic alterations of the myocardium are most severe and/or extensive, and cardiac lesions in mice injected with cell wall fragments of strain S43/100 are more severe or extensive than those in animals receiving the cell wall fragments of strain S.F. 42. No significant lesions were observed in the lungs, liver, spleen or kidneys in this work.

2. *Induction of cardiac lesions with a higher molecular weight fraction of the cell walls of strain S.F. 42 obtained enzymatically*

Most of the mice receiving repeated intraperitoneal injections of HMW developed cardiitis with the same features as that observed on injection of cell wall fragments, though the lesions seemed to be somewhat more extensive

(Table 3). Pericardial lesions were observed 2 and 7 days after the last injection, but not in tissues collected 4 weeks later. These lesions were characterized by infiltration of mononuclear cells and neutrophils into the epicardial and subepicardial tissue (Fig. 5). The myocardium showed degeneration of myofibers and focal accumulation of mononuclear cells and neutrophils. These lesions are illustrated in Figs. 6, 7, 8, 9, 10 and 11. In some lesions myofibers were no longer striated, but were highly chromatophilic and had large, swollen nuclei. In other lesions myofibers had lost their striations and appeared hyaline (Fig. 12). Granulomatous lesions were also observed in the myocardium 4 weeks after the last injection (Fig. 13). The myocardial lesions were more severe in the left ventricle, left atrium and papillary muscles of the left ventricle than in those on the right side. The endocardium showed focal accumulation of mononuclear cells (Fig. 14). The heart valves were thickened as a result of edema and infiltration of round, inflammatory cells, including plasma cells (Fig. 15). The inflammation of the valves extended to the myocardium adjacent to the roots of the valves (Fig. 16, 17 and 18). The coronary arteries and aorta appeared normal and no lesions were found in organs other than the heart.

DISCUSSION

The present work showed clearly that a higher molecular weight fraction (HMW) obtained enzymatically from the cell walls of group A streptococci induced definite cardiac lesions in mice on daily intraperitoneal injection for 4 days. The lesions induced by HMW were more extensive than those elicited by cell wall fragments in control experiments, though they differed histologically from the carditis occurring in rheumatic fever. The distribution of the cardiac lesions induced by HMW with involvement of the pericardium, myocardium, endocardium and valves, and in particular the left side of the heart, was rather similar to that seen in rheumatic fever or that induced in rabbits (Murphy, 1964). However, degenerative changes were greater and proliferative changes were much less marked in the cardiac lesions induced by HMW. No granuloma with similar histological characteristics to the Aschoff body observed in rheumatic carditis (Wagner, 1960) was induced by either HMW or cell wall fragments.

The nature of the toxic moiety of HMW responsible for induction of cardiac lesions in mice and the mechanism by which the lesions are induced are unknown. Ohanian et al. (1969) demonstrated that cell wall fragments of group A streptococci induced carditis in mice but unfragmented cell walls did not. They found that the cellular materials remained around the sites of active lesions in the heart for at least 5 weeks after injection. From these findings, they emphasized the importance of the particle size of the active principle in induction of cardiac lesions and in development of nodular skin lesions (Roberson, Schwab and Cromartie, 1960). The present work clearly indicates that soluble components of the cell walls of group A streptococci obtained by digestion with the streptolytic L-11 enzyme are as active as sonically disrupted cell wall fragments. These results do not support the hypothesis of Schwab and his coworkers that the cardiotoxic effect of streptococcal cell walls are closely related to their particle size.

As described in the previous paper, analytical data on HMW suggest that this soluble cell wall component is a complex of group specific C polysaccharide and glycopeptide. Rotta and Bednář (1969) reported induction of myocardial lesions in rabbits on intravenous injection of a peptidoglycan (mucopeptide) preparation, containing no significant amount of C-polysaccharide, isolated by extraction of group A streptococcal cell walls with hot formamide. On the contrary, Goldstein and Trung (1968) reported that a streptococcal polysaccharide prepared from cell walls by extraction with formamide, had scarcely any toxicity even at high doses when injected alone, but that it in-

duced diffuse lesions of the connective tissues in general, and the cardiovascular system in particular when injected as macromolecular "glucido-protidic" complexes. These complexes were either obtained from the cell walls or prepared artificially by coupling the polysaccharide with non-streptococcal protein. The same type of lesions was produced with a streptococcal or pneumococcal C-polysaccharide-antibody complex. Therefore, they claimed that the C-polysaccharide played some pathogenic role in streptococcal infections, though the lesions induced were not similar to those occurring in rheumatic fever. The data of Goldstein and Trung (1968) are apparently in conflict with those of Rotta and Bednář (1969). The significance of this discrepancy is unknown, and further work is required on which component of streptococcal cell walls exhibits cardiotoxicity. Further work is also required on whether the cardiotoxicity of HMW is modified or destroyed by treatment with enzymes hydrolyzing known linkages in it, and whether the cell walls or cell wall components of bacterial species other than group A *Streptococcus pyogenes* can induce cardiac lesions. Such studies should be useful to elucidate the chemical nature of the active principle responsible for cardiotoxicity and related pathogenic activities. In this connection, the possibility that the observed cardiotoxicity of HMW might be due to extraneous endotoxins should be taken into consideration, since Char and Wagner (1960) induced myocardial lesions in rabbits with meningococcal endotoxin and *Escherichia coli* lipopolysaccharide as well as with sonic extracts of group A streptococci. This possibility, however, seems unlikely because during preparative procedures great care was taken to avoid contamination of the HMW preparation with extraneous endotoxins.

The mechanism by which cardiac lesions are produced by streptococcal cell walls or their component is unknown. They may be due to a toxic, irritative mechanism and/or an immunologic mechanism as proposed by Goldstein and Trung (1968). Abdula and Schwab (1965) reported that the sera of nearly all normal rabbits tested reacted with peptidoglycan preparations isolated from the cell walls of streptococci of groups A, C, and D, and other bacterial species (*Staphylococcus albus*, *Bacillus cereus* and *Chromobacterium violaceum*), and that this represented a "natural" immunological response against an ubiquitous antigenic moiety common to a variety of microbial peptidoglycans. Schwab and Brown (1968) also showed that 7 days after intraperitoneal injection of 10 mg of group A streptococcal cell walls mice had developed cell wall agglutinating antibodies, and that these were maintained at a rather constant level in the circulation for at least 24 weeks. These observations, together with findings of Goldstein and Trung (1968), suggest that an immunological mechanism may be involved in development of cardiotoxic responses, especially late responses, against streptococcal cell walls or HMW. However, this does not exclude the participation of a direct toxic effect of the cell walls or HMW in induction of cardiac lesions. Schwab and Ohanian (1968) considered that a peptidoglycan had a direct irritating effect on host tissues, and that C polysaccharide protected the peptidoglycan from the defense mechanisms of the host, so that its irritating effect persisted.

The cardiac lesions induced by HMW seem to be more severe and extensive than those induced by sonically disrupted cell wall fragments. This may be because HMW can reach the heart more readily than particulate fragments, since it is a true solution. Ohanian et al. (1969) suggested that the relative ineffectiveness of a suspension of large fragments compared with small fragments of cell walls might reflect the poor distribution and location of the former at a critical site, or a difference due to processing and elimination of different size particles.

At present no conclusion can be drawn about the chemical nature of the cardiotoxic principle in streptococcal cell walls or its mechanism of action on cardiac tissue. Comparative investigations on the pathogenic activities of the cell

walls of a variety of bacterial species and studies on the effects of various hydrolytic enzymes on the biological activities of bacterial cell walls are in progress.

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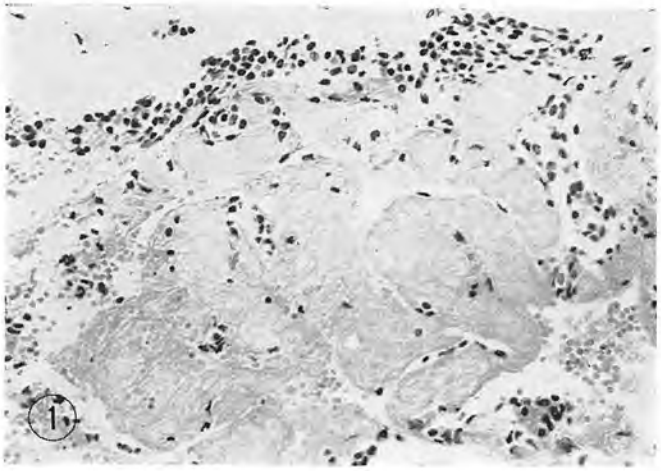

FIGURE 1. *Infiltration of the epicardium with inflammatory cells. Section from a mouse sacrificed 4 weeks after the last injection of cell wall fragments (strain S.F. 42).* × *100.*

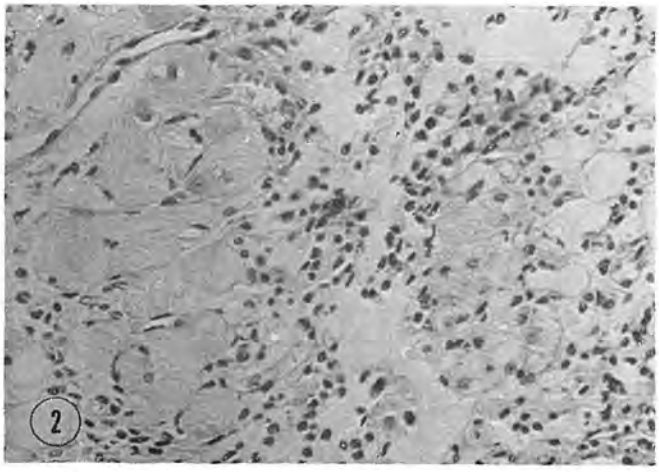

FIGURE 2. *Granulomatous lesions of the myocardium and degeneration of myofibers. Section from a mouse sacrificed 4 weeks after the last injection of cell wall fragments (strain S43/100).* × *100.*

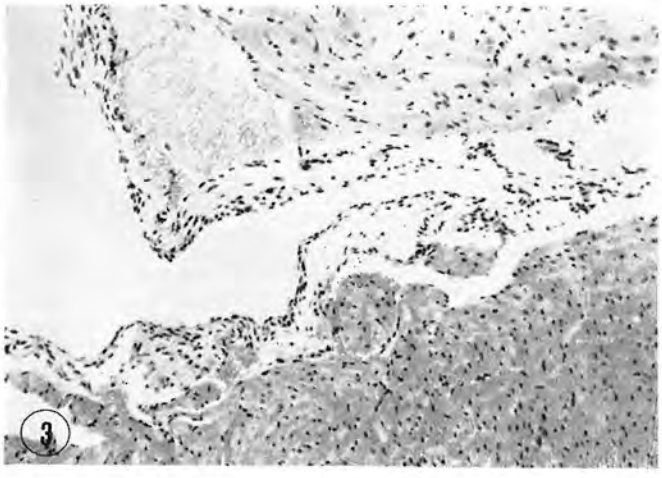

FIGURE 3. *Slight edematous changes of the mitral valve. Section from a mouse sacrificed 2 weeks after the last injection of cell wall fragments (strain S43/100).* × *50.*

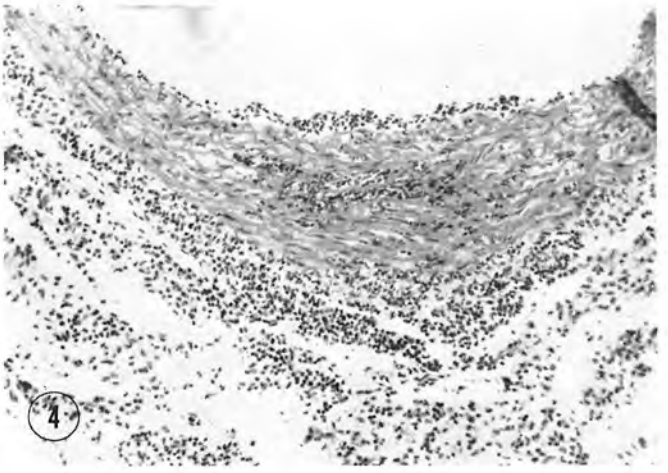

FIGURE 4. *Infiltration in and around the aorta with round cells. Section from a mouse sacrificed 4 weeks after the last injection of cell wall fragments (strain S.F. 42).* × *50.*

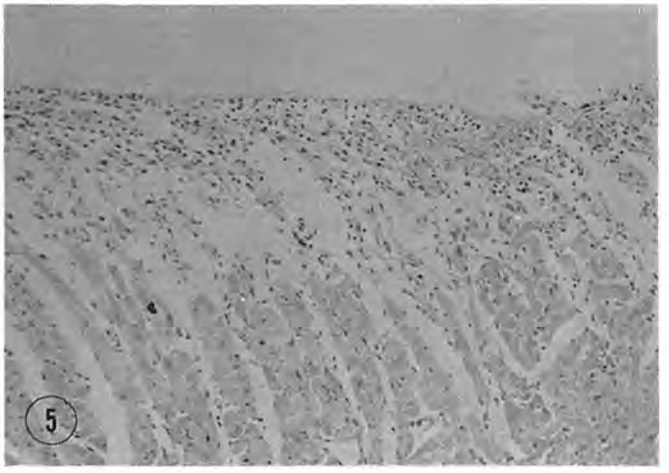

FIGURE 5. *Localized infiltration of the epicardium with round cells. Section from a mouse sacrificed 2 days after the last injection of HMW.* × *50.*

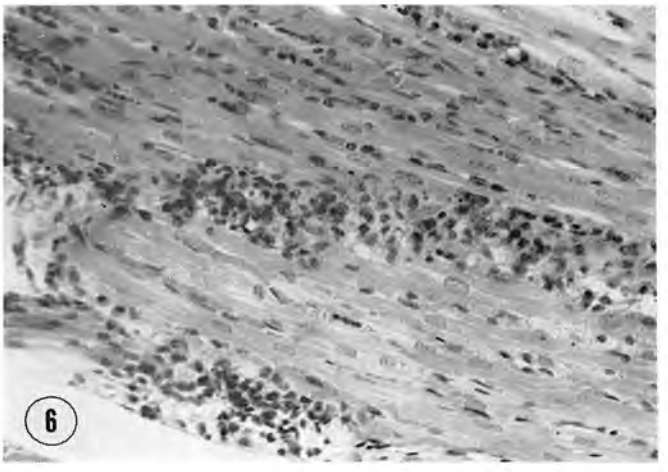

FIGURE 6. *Infiltration of mononuclear cells and neutrophils into the myocardium. Section from a mouse sacrificed 1 week after the last injection of HMW.* × *100.*

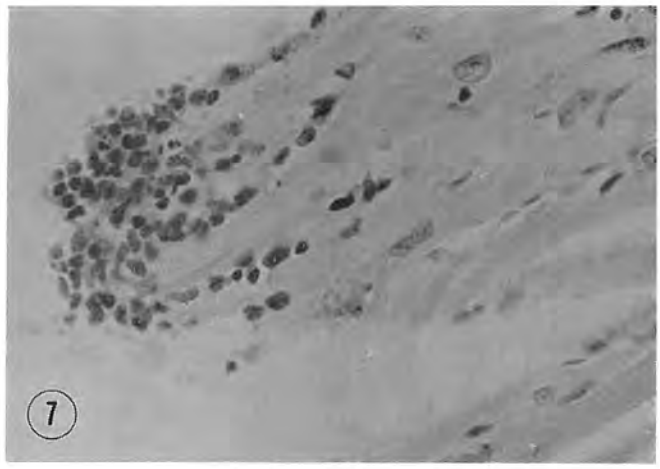

FIGURE 7. *Focal collection of mononuclear cells and neutrophils in the myocardium. Section from a mouse sacrificed 1 week after the last injection of HMW.* × *200.*

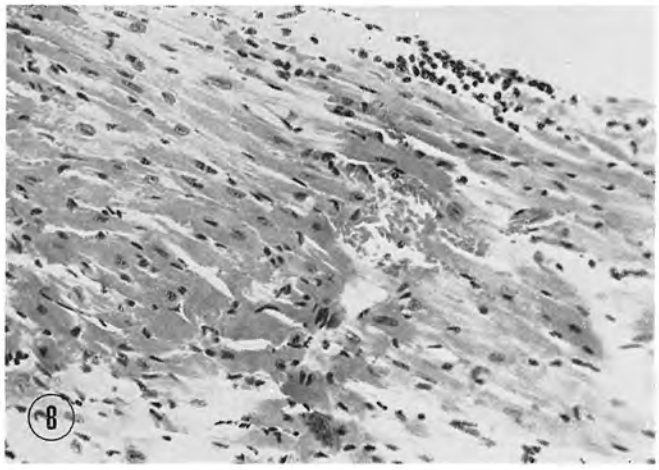

FIGURE 8. *Infiltration of mononuclear cells and neutrophils, and degenerative changes in the mycardium. Section from a mouse sacrificed 4 weeks after the last injection of HMW.* × *100.*

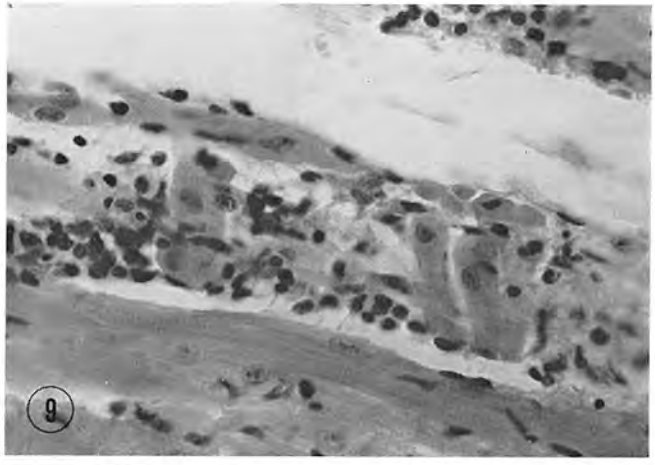

FIGURE 9. *Degenerative changes, and infiltration of mononuclear cells and neutrophils into the myocardium. Section from a mouse sacrificed 2 days after the last injection of HMW.* × *200.*

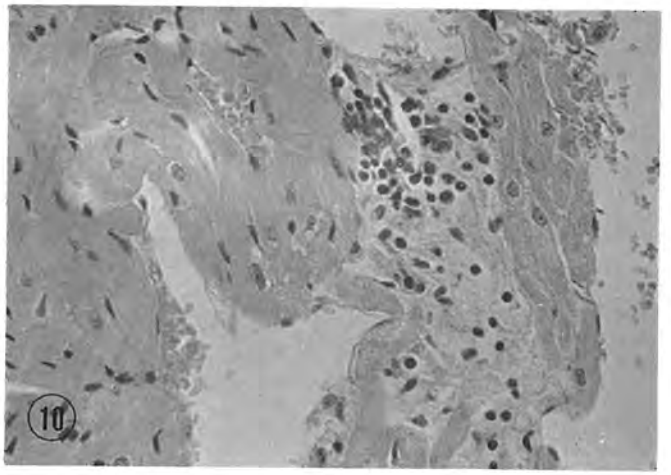

FIGURE 10. *Degenerative changes, and infiltration of mononuclear cells and neutrophils into the left papillary muscle. Section from a mouse sacrificed 4 weeks after the last injection of HMW.* × *100.*

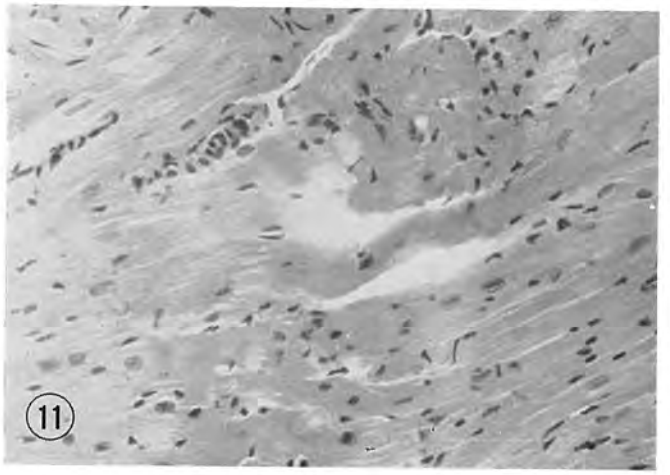

FIGURE 11. *Chromatophilia and fragmentation of myofibers with slight cellular infiltration. Section from a mouse sacrificed 4 weeks after the last injection of HMW.* × *100.*

FIGURE 12. *Waxy degeneration of myofibers. Section from a mouse sacrificed 1 week after the last injection of HMW.* × *50.*

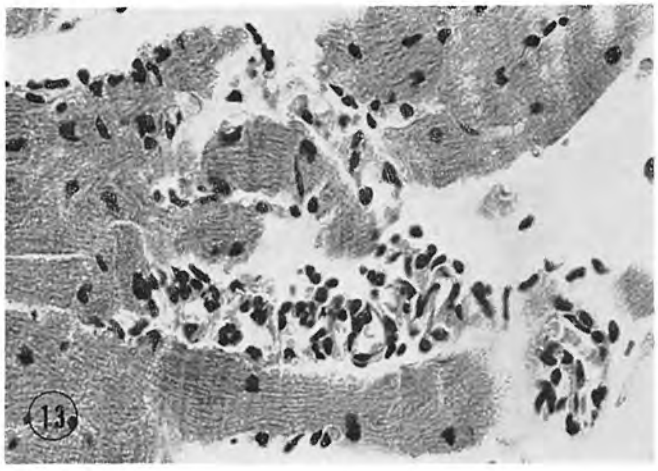

FIGURE 13. *Granulomatous lesions in the myocardium. Section from a mouse sacrificed 4 weeks after the last injection of HMW.* × *200.*

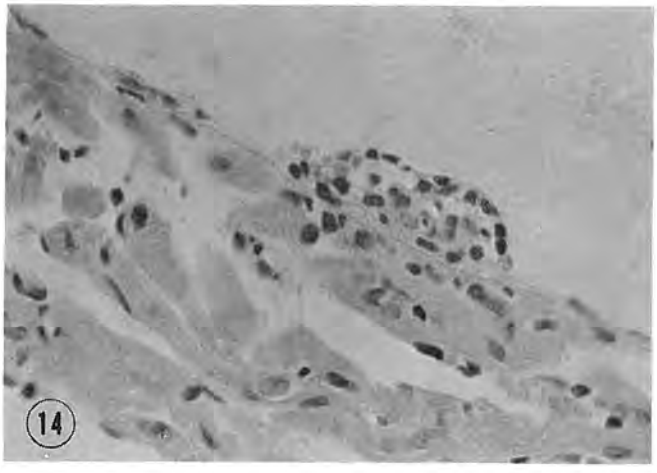

FIGURE 14. *Mural accumulation of mononuclear cells and neutrophils in the endocardium. Section from a mouse sacrificed 4 weeks after the last injection of HMW.* × *200.*

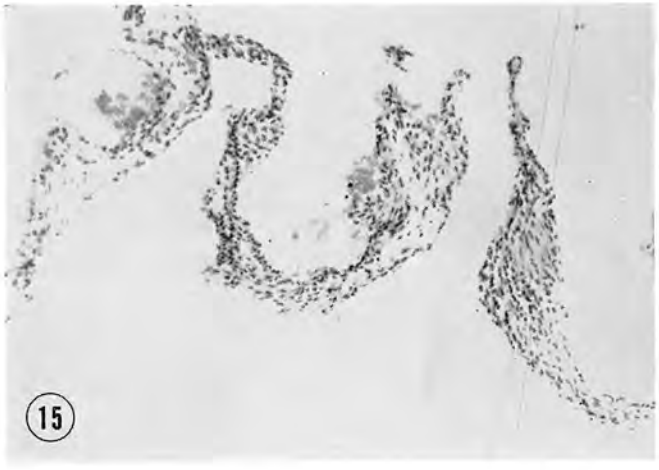

FIGURE 15. *Edematous changes of the mitral valve with infiltration of round cells. Section from a mouse sacrificed 1 week after the last injection of HMW.* × *50.*

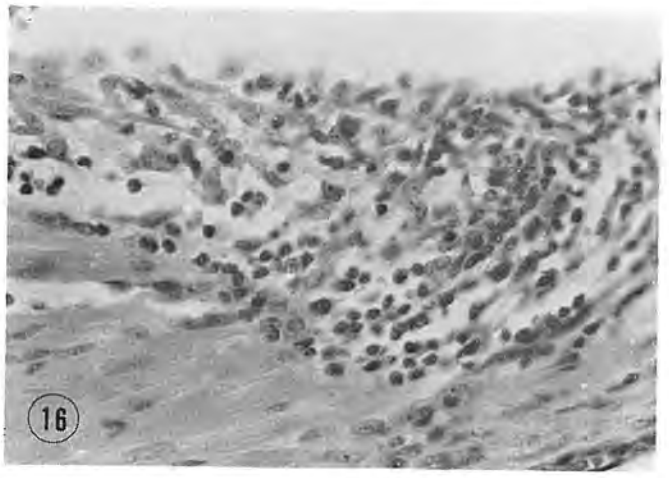

FIGURE 16. *Granulomatous lesions of the root of the mitral valve and adjacent myocardium. Section from a mouse sacrificed 2 days after the last injection of HMW.* × *200.*

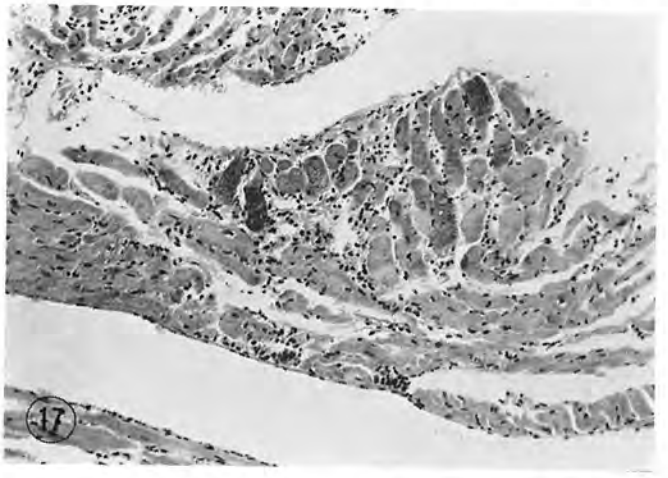

FIGURE 17. *Infiltration of inflammatory cells and degenerative changes in the root of the mitral valve and adjacent myocardium. Section from a mouse sacrificed 1 week after the last injection of HMW.* × *50.*

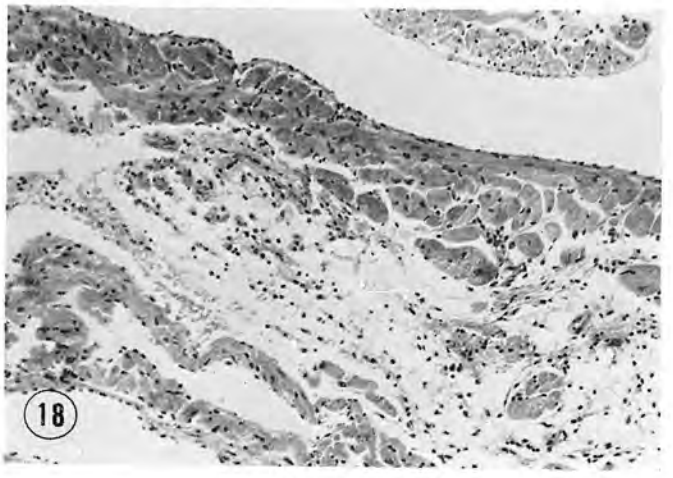

FIGURE 18. *Edematous and degenerative changes of the root of the mitral valve and adjacent myocardium. Section from a mouse sacrificed 1 week after the last injection of HMW.* × *50.*