COXSACKIE VIRUS B_4 PANCARDITIS IN CYNOMOLGUS MONKEYS RESEMBLING RHEUMATIC HEART LESIONS*

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Received for publication June 15, 1967

STUDIES in this laboratory have shown that mural and valvular endocarditis can be consistently produced in mice with coxackie B_4 virus (Burch, De Pasquale, Sun, Mogabgab and Hale, 1966*a*; Burch, De Pasquale, Sun, Hale and Mogabgab, 1966*b*; Sun, Burch, Colcolough, De Pasquale and Sohal, 1967). A recent survey of the incidence of coxsackie B_4 virus infection in human hearts obtained at routine autopsy adds support to the hypothesis that some cardiotropic viruses may play important role in the genesis of chronic heart diseases (Burch, Sun, Colcolough, Sohal and De Pasquale, 1967). Because of the implications of these findings relative to the etiology of valvular heart disease in man, further investigations on the nature of the cardiac lesions produced by coxsackie virus B_4 were conducted in cynomolgus monkeys. Earlier observations had indicated that there were some similarities between the valvular lesions observed in chronically infected cynomolgus monkeys and rheumatic endocarditis in man (DePasquale, Burch, Sun, Hale and Mogabgab, 1966). The purpose of this paper is to describe further the nature of the valvular and myocardial lesions in the infected monkeys employing immunofluorescent antibody and electron microscopic techniques, as well as conventional histological studies.

MATERIALS AND METHODS

Monkeys.—Fifteen monkeys were obtained from Asiatic Animal Imports, San Francisco, California and were observed for 30 days in the Vivarium before use.

Virus stock.—The coxsackie B_4 virus used in these experiments was recovered by Kibrick and Benirschke (1958) from a fatal case of encephalohepatomyocarditis in a 10-day-old infant. The virus obtained as a monkey kidney culture passage strain was prepared for inoculation in rhesus monkey kidney cultures. Control culture fluid from virus-free monkey kidney cell medium was also obtained. The virus and control fluids were stored at $-65°$.

Inoculation of virus and collection of tissues.—In this study 15 cynomolgus monkeys were used. Eleven animals were inoculated i.v. with 0·3 ml. of monkey kidney culture fluid containing 10^5 $TCID_{50}$ of coxsackie virus B_4. The monkeys were killed by anaesthesia 51–200 days after inoculation. The hearts were excised and inspected grossly for valvular lesions. Random pieces of valvular, mycardial and pericardial tissues were prepared for: (1) frozen sections for fluorescent antibody technique, (2) paraffin embedding for routine histology and (3) electron microscopy.

Four monkeys were inoculated i.v. with 0·3 ml. of virus-free monkey kidney culture fluid. They were killed at similar time periods after injection and their cardiac tissues were processed concurrently with those of the infected animals.

* Supported by grants from the United States Public Health Service, Rowell A. Billups Fund for Research in Heart Disease and the Rudolph Matas Fund for the Kate Prewitt Hess Laboratory.

Immunofluorescent studies.—Coxsackie virus B_4 antiserum was prepared in rabbits by 2 i.v. injections of 10 ml. of the virus stock solution at 2-week intervals. The antiserum was fractionated and conjugated with fluorescein isothiocyanate according to the method of Coon and Kaplan (1950). Cryostat sections (6μ) of heart were stained for viral antigen by the direct immunofluorescent antibody technique (Coon and Kaplan, 1950). Staining specificity for coxsackie virus B_4 antigen was checked: (1) by using fluorescein-conjugated rabbit antisera prepared against virus-free monkey kidney cell culture fluid for control staining and (2) by pretreatment with unconjugated antiserum, followed by the application of conjugated antiserum (Cherry, Goldman, Carski and Moody, 1960).

Histological studies.—Paraffin sections of heart muscle and valves were stained with hematoxylin and eosin.

Electron microscopy.—Small (1 cu. mm.) blocks of myocardium were fixed at 4° in phosphate-buffered osmium tetroxide for 75 min. The tissues were dehydrated in ethanol and embedded in Maraglas. Ultrathin sections were cut on an LKB Ultratome and stained with uranyl acetate and lead citrate. They were then observed with a Siemens Elmiskop I.

RESULTS

The gross observations are summarized in the Table. A chronic valvulitis showing fibrous thickening of the valves with or without verrucous formation was noted in the mitral and/or aortic valves in 8 of the 11 infected animals (Figs. 1 and 2). A typical instance of mitral stenosis with adhesion of commissures and contracture of chordae tendineae was observed in one monkey 200 days after the initial infection (Fig. 3).

TABLE.—*Valve Lesions in Cynomolgus Monkeys Inoculated with Coxsackie Virus B_4*

Animal No.	Days after inoculation	Valve lesions
Experimental		
1	51	Verrucous lesion of mitral valve
2	51	Verrucous lesion of aortic valve
5	74	Verrucous lesions of mitral and aortic valves
6	178	Verrucous lesion of mitral valve
7	185	Cicatricial thickening of mitral valve with commissural adhesions
8	199	None
9	200	Mitral stenosis with commissural adhesion and contracture of chordae tendineae
10	90	Cicatricial thickening of mitral valve
11	92	None
13	120	None
14	120	Cicatricial thickening of mitral valve
Control		
3	51	None
4	74	None
12	92	None
15	120	Thickening of mitral valve

Verrucous lesions of the valves, when present, were characterized by a focal scar located on the free margin of the valve leaflet with a smooth layer of endothelium covering its surface. When present on the aortic valve, it was located on the ventricular aspect of the cusps. No gross lesions were found on the tricuspid or pulmonary valves of any of the infected animals.

Histologically, the valvular lesions showed a chronic inflammatory process. Fibrous scarring and a mononuclear cell infiltrate were the predominant features

(Fig. 4). Fibrinoid degeneration with hyalinization of the collagen fibres was occasionally encountered. Coxsackie B_4 virus antigen was demonstrated by the immunofluorescent method in 3 valves which showed gross lesions (Fig. 5). Two additional valves (a mitral and a pulmonary) from 2 separate monkeys also contained viral antigen but showed no gross lesions (Fig. 6). A chronic mural endocarditis with inflammatory cell infiltrate and detectable coxsackie B_4 antigen within the endothelial cells was observed in 2 monkeys (Fig. 7).

Scattered areas of focal myocarditis were seen in all 11 infected monkeys. The lesions were noted to be in varying stages of development. Early changes consisted of segmental disintegration of the muscle fibres associated with an infiltration of numerous small round and polymorphonuclear cells (Fig. 8). In the more chronic stages, the lesions were fairly well circumscribed and exhibited a fusiform nodular pattern composed of giant multi-nucleated myocytes and mononuclear inflammatory cells (Fig. 9).

Scattered areas of perivascular inflammation were frequently seen in the chronically infected animals (Fig. 10). These lesions consisted of disrupted collagen fibres and a few mononuclear inflammatory cells. Most of the myocytes in the vicinity of the lesions showed large, bizarre-shaped, hyperchromatic nuclei (Fig. 10). The larger branches of the coronary arteries of one infected monkey displayed regional arteritis (Fig. 11).

The immunofluorescent procedure usually demonstrated coxsackie virus B_4 antigen to be within the perinuclear cytoplasm of the myofibres when present. The mononuclear cells also frequently contained fluorescent antigen within their cytoplasm (Fig. 12).

Scattered chronic inflammation of the epicardium with adhesion formation was frequently seen in the infected monkeys. Viral antigen was detected in most instances within the inflammatory or epicardial tissue cells in these areas (Fig. 13).

Slight fibrotic thickening of the mitral valve was noticed in one of the control monkeys. Histologically a nonspecific fibrosis was seen within the substance of the leaflets. Coxsackie viral antigen was not detected within this area by the immunofluorescent technique. There were no microscopic myocardial lesions in the control animals as described above for the infected animals.

Electron microscopic examination of the myocardium of the infected monkeys demonstrated ultrastructural changes consistent with focal myofibre damage (Fig. 14). Mitochondria of the affected myocytes were reduced in number, swollen and contained disrupted cristae with a decrease in the electron density of their matrix (Figs. 15, 16). The extent of damage of the myofibres varied. In some myocytes the myofibrils lost their periodic bands, whereas in others, extensive dissolution of the myofibrils was seen (Figs. 14, 15, 16). The degenerative areas were occupied by loose ground substance, lysosomes, glycogen, randomly dispersed vesicles and swollen mitochondria (Fig. 15).

The tubules of the sarcoplasmic reticulum of the damaged myofibres were swollen and dilated (Fig. 14). In addition to the smooth-surfaced sarcoplasmic reticulum, several membranes with ribosomal particles on their surface could be identified. These membranes were seen most frequently between the myofibrils.

Lipid droplets were noticeable in some regions of the myocardium. The droplets were usually associated with mitochondria and appeared to be bound by a single membrane. Several extremely large vesicles bound by double membranes, ranging up to several microns in diameter, were occasionally seen among the

degenerating myofibres (Fig. 16). Osmiophilic particles were occasionally present inside the vesicles. The intercellular spaces at the region of fascia adherens of the intercalated discs were widened. There was no significant ultrastructural change in the nuclei.

EXPLANATION OF PLATES

FIG. 1.—Chronic mitral valvulitis of a cynomolgus monkey inoculated with coxsackie B_4 virus 120 days before autopsy. Note the fibrotic thickening of the mitral valve leaflet and chordae tendineae.

FIG. 2.—Chronic mitral valvulitis of a cynomolgus monkey inoculated with coxsackie B_4 virus 185 days before autopsy. Note the fibrotic thickening of the mitral valve leaflet with verrucous formation at the free margin.

FIG. 3.—Chronic mitral valvulitis with mitral stenosis in a cynomolgus monkey inoculated with coxsackie B_4 virus 200 days before autopsy. Note cicatricial thickening of the valve with adhesion of commissures and contracture and thickening of the chordae tendineae.

FIG. 4.—Chronic viral valvulitis of an aortic valve of a cynomolgus monkey infected with coxsackie B_4 virus 51 days before autopsy. Note the fibrous scarring with infiltrating mononuclear cells. H. and E. ×90.

FIG. 5.—Mitral valve of a cynomolgus monkey (from same valve shown in Fig. 1) infected with coxsackie B_4 virus 120 days before autopsy. Note the intracytoplasmic immunofluorescent antibody staining specific for coxsackie B_4 virus in the stromal cells of the valve. Direct immunofluorescent stain. ×360.

FIG. 6.—Pulmonary valve of a cynomolgus monkey infected with coxsackie B_4 virus 90 days before autopsy. Note the intracytoplasmic immunofluorescence specific for coxsackie B_4 virus in mononuclear cells of the stroma. Direct immunofluorescent stain. ×360.

FIG. 7.—Mural endocardium of the left ventricle (from same heart shown in Fig. 1) of a cynomolgus monkey infected with coxsackie B_4 virus 120 days before autopsy. Note the specific intracytoplasmic immunofluorescence in the surface endothelial cell. Direct immunofluorescent stain. ×360.

FIG. 8.—Coxsackie B_4 viral myocarditis in a cynomolgus monkey 51 days after i.v. inoculation. Note the segmental disintegration of the muscle fibres associated with numerous infiltrating cells and the deformed myocyte exhibiting an " owl-eyed " nucleus (arrow). H. and E. ×360.

FIG. 9.—Coxsackie B_4 viral myocarditis in cynomolgus monkey 185 days after i.v. inoculation. Note the fusiform nodular lesion exhibiting giant multi-nucleated myocytes (small arrow). Nuclei of the adjacent muscle fibres show characteristic aggregation of the chromatin granules resembling Anitschkow myocyte (big arrows). H. and E. ×360.

FIG. 10.—Coxsackie B_4 viral myocarditis in a cynomolgus monkey 120 days after infection. Note the myocardial lesion centred about a small blood vessel with damaged and deformed surrounding myocytes (arrow). H. and E. ×360.

FIG. 11.—Coxsackie B_4 viral arteritis of a coronary artery of a cynomolgus monkey 185 days after infection. Note an area of necrosis in the vascular wall (arrows). H. and E. ×360.

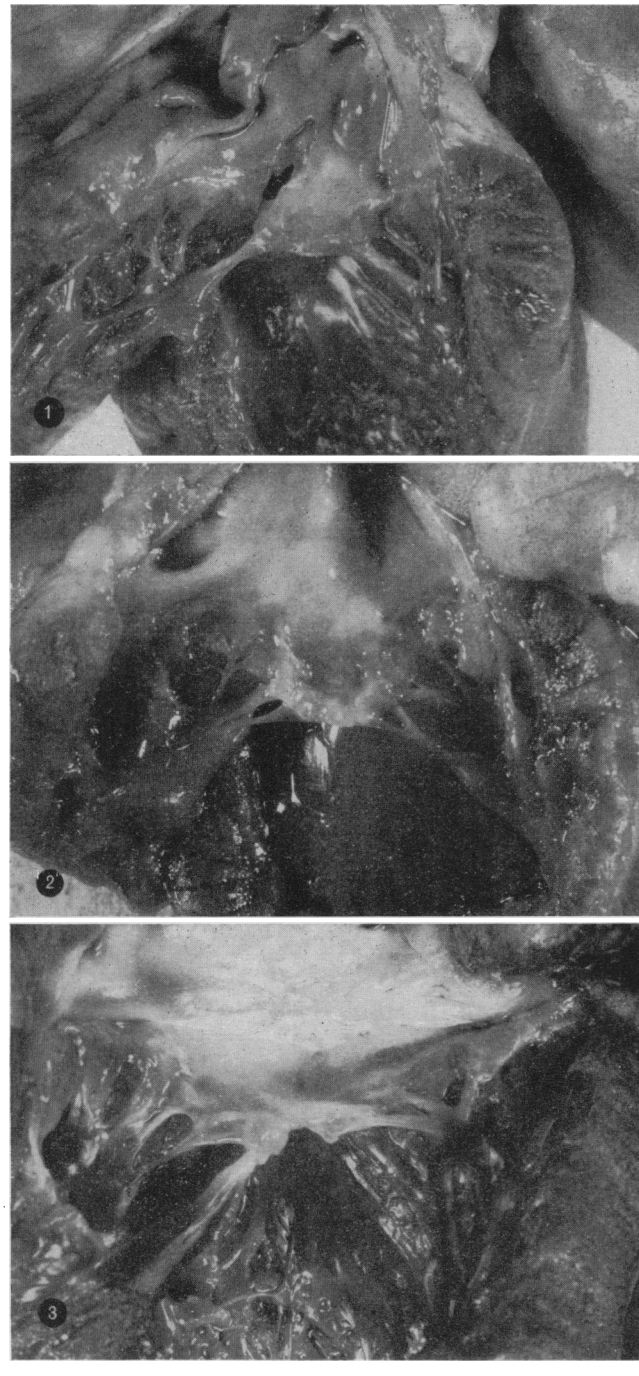
FIG. 12.—Myocardium of a cynomolgus monkey infected with coxsackie B_4 virus 92 days before autopsy. Note the specific immunofluorescence in the cytoplasm of a muscle fibre and a mononuclear cell. Direct immunofluorescent stain. ×385.

FIG. 13.—Myocardium with a thickened portion of epicardium from a cynomolgus monkey infected with coxsackie B_4 virus 92 days before autopsy. Note the specific intracytoplasmic immunofluorescence in the cells of the thickened epicardium as well as in the myofibre. Direct immunofluorescent stain. ×385.

FIG. 14.—An electron micrograph of 2 adjacent cardiac cells separated by an intercalated disc (ID). The 2 cells are at different degenerative stages. The one on the right has relatively intact myofibres; the cell on the left shows degeneration in the peripheral region and the myofibrils have undergone extensive degeneration. The sarcoplasmic reticulum in both cells is somewhat swollen. (From a monkey infected 51 days before autopsy.) ×36,000.

FIG. 15.—An electron micrograph of the peripheral region of a degenerating myofibre. The area subjacent to the sarcolemma (S) is occupied by loose ground substance, vesicles (V), glycogen (G), lysosomes (L) and swollen mitochondria (M). (From a monkey infected 51 days before autopsy.) ×36,000.

FIG. 16.—Electron micrograph of a damaged myofibre. Note the swollen mitochondria with irregularly oriented cristae. Two large vesicles (V) with double limiting membranes are present. These vesicles probably result from cell necrosis. A few glycogen-like particles (G) are present in the vesicles. (From a monkey infected 90 days before autopsy.) ×24,000.



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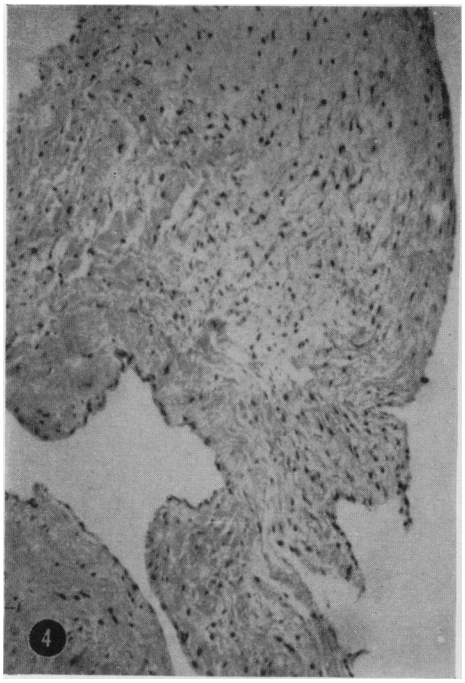

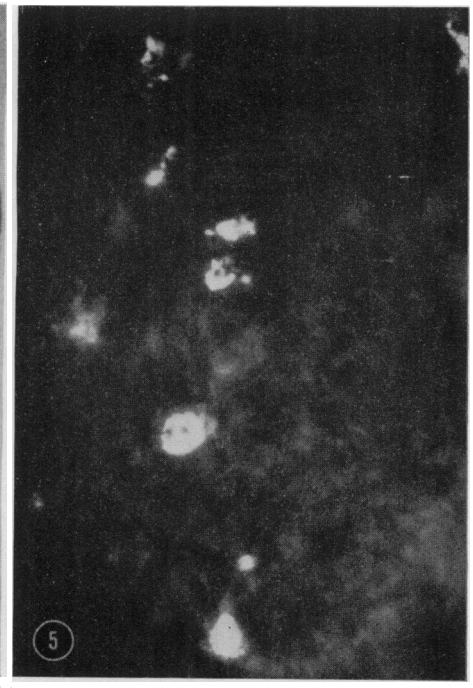

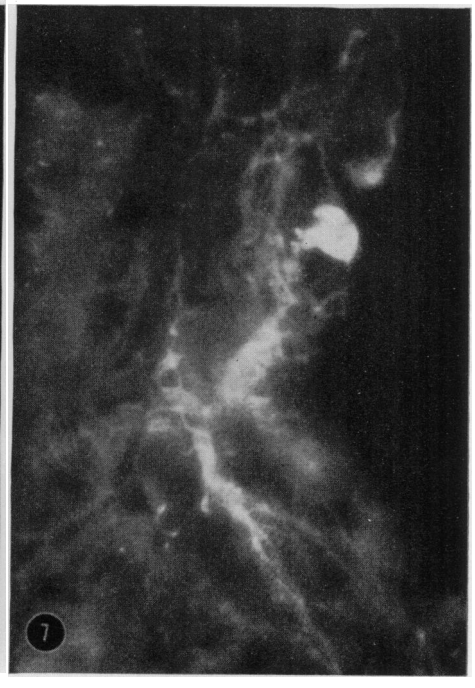

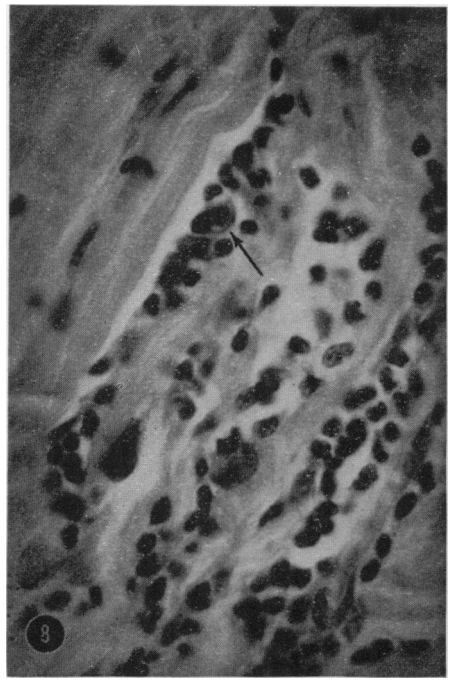
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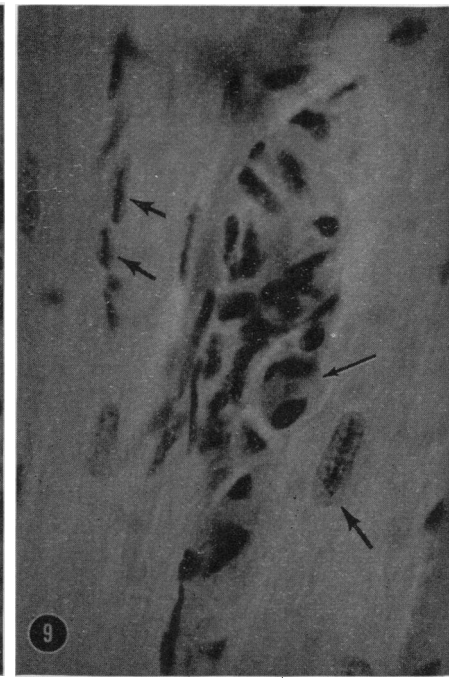
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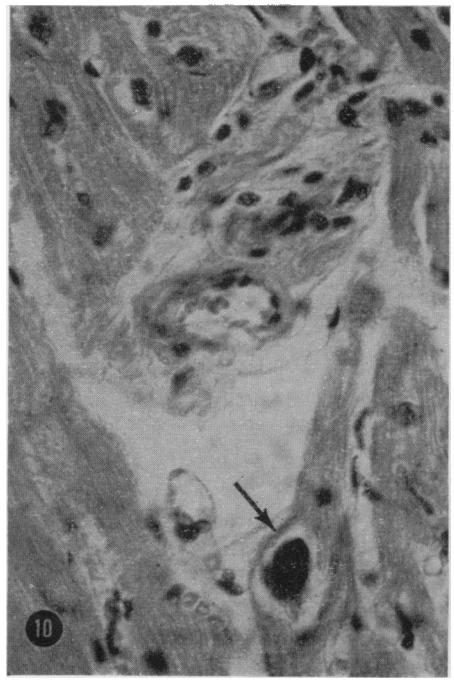
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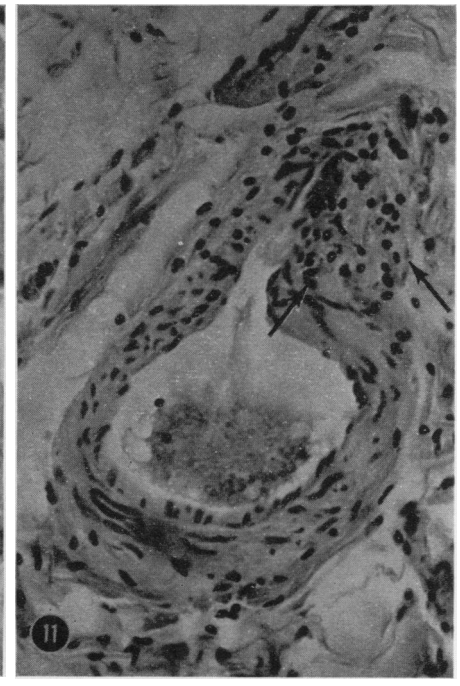
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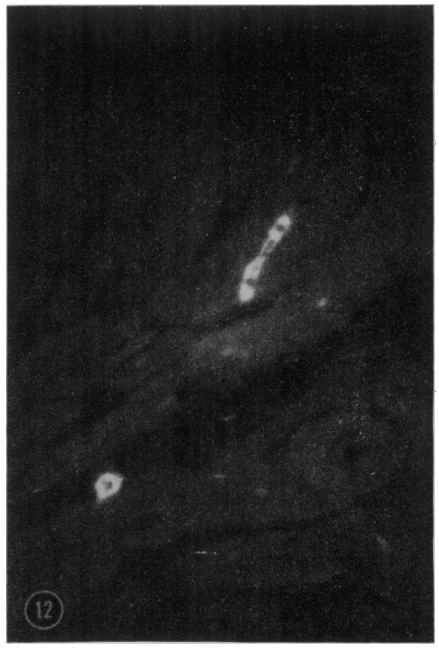
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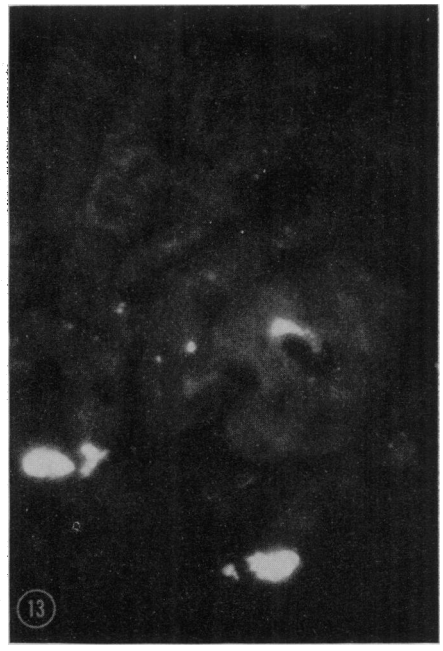
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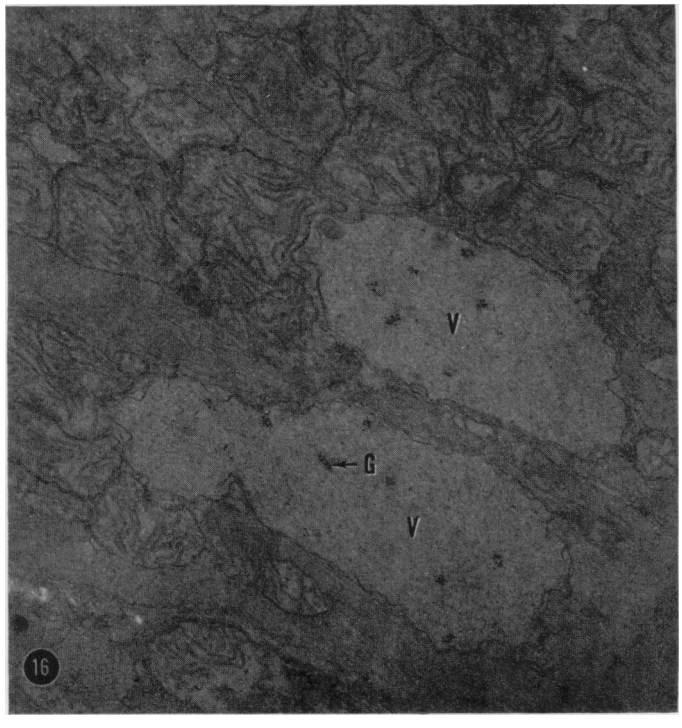
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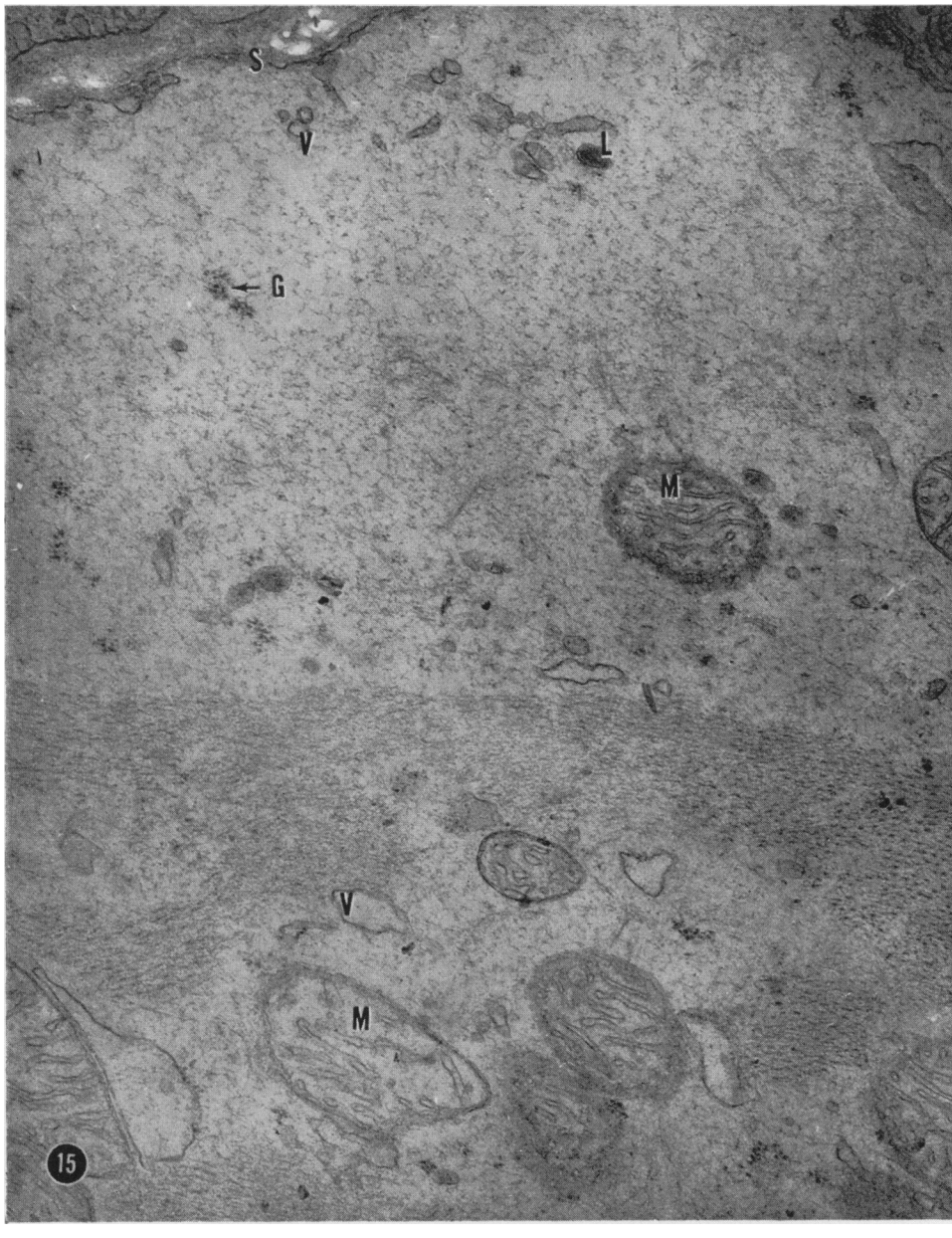

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DISCUSSION

Coxsackie B viral myocarditis and pericarditis has been studied within the past decade, in both man (Burch *et al.*, 1967; Smith, 1966) and the experimental animal (Dempster, Grodum and Spencer, 1966; Rabin, Hassan, Jenson and Melnick, 1964; Sohal, Sun, Burch and Colcolough, 1967). Coxsackie B viral endocarditis and valvulitis has received little attention (Burch *et al.*, 1966*a*, *b*; Lou, Wenner and Kamitsuka, 1961*a*, *b*). Previous studies have indicated that acute and chronic inflammatory changes associated with coxsackie B viral antigen detectable by immunofluorescence could be consistently demonstrated in a significant proportion of valves of experimentally infected animals (Burch *et al.*, 1966*a*, *b*; DePasquale *et al.*, 1966; and Sun *et al.*, 1967). More recently, coxsackie B viral antigen has been found at post mortem in a number of valves of human hearts at routine autopsy (Burch *et al.*, 1967).

The term endocarditis has been used almost universally to denote the valvular lesion of rheumatic fever (Gould, 1960). However, in our present studies with coxsackie viral infection lesions of both mitral and aortic valves were found to be grossly similar to that of rheumatic valvulitis in man. Particularly interesting is an instance in a cynomolgus monkey of chronic mitral valvulitis with scarring and deformity of the valve, adhesion of the commissures and contracture of chordae tendineae producing a " mitral stenosis " similar to that seen in some forms of chronic rheumatic valvular disease in man. The characteristic Aschoff nodules were not seen in histological sections of these valves; but mononuclear cells, hyalinized collagen fibres and immunofluorescent antigen specific for coxsackie B_4 virus were noted in a significant proportion of the involved valves.

The myocardial lesions of coxsackie myocarditis associated with multinucleated giant myocytes are quite similar histologically to the Aschoff body. The myocytes bearing large hyperchromatic nuclei in the vicinity of the chronic lesions centred about small blood vessels is a further similarity to some stages of rheumatic carditis. Since the coxsackie viral antigen was frequently found in the perinuclear zone of the myocytes, the nuclear deformity may be related to viral infestation within the myocytes. These altered myocytes may possibly be the origin of Anitschkow's giant cells of Aschoff bodies (Murphy, 1960). However, it is still controversial whether large cells of Aschoff bodies are derived from cardiac and smooth muscle cells or connective tissue cells (Lannigan and Zakis, 1963). Focal disintegration of myocardial fibres usually seen in the earlier phase of rheumatic myocarditis in man was also found in the infected monkeys with detectable specific coxsackie viral antigen inside the sarcoplasm.

The electron microscope is of limited value in studying the scattered lesions in the myocardium observed with light microscopy. Possibly because of this, we have not seen any cells at the ultrastructural level resembling Aschoff cell as described by Lannigan and Zakis (1963). However, the ultrastructural alterations of the myocardial fibres showing various degenerative changes ranging from vacuolation to almost complete loss of myofibrils in the infected monkeys are entirely compatible with findings reported by these authors.

The mechanism whereby the coxsackie virus produces pancarditis is unknown. However, the demonstration of the specific viral antigen in the affected tissue indicates the direct relationship of the virus to the cardiac disease.

Rheumatic fever in man is well known to be preceded by an upper respiratory

infection. Kendall, Cook and Stone (1960) reported that coxsackie virus infection may produce febrile coryza or bronchitis in children. However, the evidence for incriminating haemolytic streptococci as the aetiologic factor in rheumatic fever has been accumulating for many years. Pearse (1960) suggested that the haemolytic streptococcus may possibly pave the way for some unknown virus to attack the heart tissue. Freud, Rook and Brunhofer (1950) drew attention to the risk of reactivating rheumatic fever by vaccination, which was thought to be a source of infection. In a previous report from this laboratory, it was postulated that some instances of acute and chronic valvulitis in man may be viral in origin (Burch and DePasquale, 1964). A relatively large number of patients considered to have rheumatic valvular disease give no history of rheumatic fever. It seems possible that cardiotropic viruses like the coxsackie B group may play an important role in the genesis of rheumatic heart disease or a similar disease state (Burch *et al.*, 1967).

SUMMARY

A coxsackie B_4 virus pancarditis resembling rheumatic pancarditis in man is described in cynomolgus monkeys.

A typical instance of mitral stenosis with adhesion of commissures and contracture of chordae tendineae is presented.

Aschoff body-like lesions with multi-nucleated myocytes as well as a type of chronic fibrotic lesion centred about a small blood vessel with myocytes resembling Anitschkow's cell are shown in the myocardium of the infected monkeys.

The specific coxsackie B_4 antigen was detected in the valves, the mural endocardium, the myocardium and the epicardium of the infected monkeys by the direct immunofluorescent technique.

The discovery of these characteristic lesions in the coxsackie B_4 virus infected monkeys may assist in a better consideration of human rheumatic heart lesions. A type of cardiotropic virus, coxsackie B group at least, may play an important role in the genesis of rheumatic heart disease or a disease state of the heart which is quite similar to it.

REFERENCES

Burch, G. E. and DePasquale, N. P.—(1964) *Am. Heart J.* **67,** 721.

Burch, G. E., DePasquale, N. P., Sun, S. C., Mogabgab, W. J. and Hale, A. R.—(1966*a*) *Science* **151,** 477.

Burch, G. E., DePasquale, N. P., Sun, S. C., Hale, A. R. and Mogabgab, W. J.—(1966*b*) *J. am. med. Ass.*, **196,** 394.

Burch, G. E., Sun, S. C., Colcolough, H. L., Sohal, R. S. and DePasquale, N. P.—(1967) *Am. Heart J.*, **74,** 13.

Cherry, W. B., Goldman, M., Carski, T. R. and Moody, M. D.—(1960) 'Fluorescent antibody technics in the diagnosis of communicable disease,' Washington (U.S. Public Health Service), Publication 729.

Coon, A. H. and Kaplan, M. H.—(1950) *J. exp. Med.* **91,** 1.

Dempster, G., Grodum, E. I. and Spencer, W. A.—(1966) *J. Cell Physiol.* **67,** 433.

DePasquale, N. P., Burch, G. E., Sun, S. C., Hale, A. R. and Mogabgab, W. J.—(1966) *Am. Heart J.*, **71,** 678.

Freud, P., Rook, G. D. and Brunhofer, A.—(1950) *J. Pediat.* **36,** 635.

Gould, S. E.—(1960) ' Pathology of the Heart ', Ed. 2. Springfield (C. C. Thomas).

Kendall, E. J., Cook, G. T. and Stone, D. M.—(1960) *Br. med. J.* 5207, 1180.

Kilbrick, S. and Benirschke, K.—(1958) *Pediatrics*, **22,** 857.

LANNIGAN, R. AND ZAKIS, S.—(1963) *Nature Lond.*, **198,** 898.
LOU, T. Y., WENNER, H. A. AND KAMITSUKA, P. S.—(1961*a*) *Arch. ges. Virusforsch.* **10,** 425.—(1961*b*) *Arch. ges. Virusforsch.*, **10,** 451.
MURPHY, G. E.—(1960) *Medicine* **39,** 289.
PEARSE, J. M.—(1960) *Circulation*, **21,** 448.
RABIN, E. R., HASSAN, S. A., JENSON, A. B. AND MELNICK, J. L.—(1964) *Am. J. Path.* **44,** 775.
SMITH, W. G.—(1966) *Br. Heart J.* **28,** 204.
SOHAL, R. S., SUN, S. C., BURCH, G. E. AND COLCOLOUGH, H. L.—(1967) *Nature Lond.*, **215,** 312.
SUN, S. C., BURCH, G. E., COLCOLOUGH, H. L., DEPASQUALE, N. P. AND SOHAL, R. S.—(1967) *Proc. Soc. exp. Biol. Med.*, **125,** 157.