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HEPATITIS IN MICE INFECTED WITH COXSACKIE VIRUS B_1*

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Summary.—The livers of mice of different ages were readily damaged by Coxsackie virus B_1 infection. The severity of liver damage decreased as the age of the mice increased. Coxsackie B_1 viral crystals were not found in the damaged liver cells in spite of severe pathological changes of the liver, both histologically and electron microscopically, and even though characteristic crystal formation was observed in the pancreas of three of these same animals. Nevertheless, the hepatic damage was considered to be due to direct viral invasion of the hepatic cells. This injury was followed by a variety of degenerative and necrotic processes displaying somewhat characteristic morphological manifestations. The severe hepatic infection produced in the newborn mice resulted in their death from a rather fulminating illness, whereas in the older mice there was recovery from mild to moderate hepatic injury with cellular regeneration by the fourth day after viral inoculation. The experimental preparation used here provides an excellent means for the study of the processes of injury and healing of the liver infected with a virus that is also infectious for man.

Viral hepatitis caused by Coxsackie B viruses has been reported in patients (Fechner, Smith and Middlekamp, 1963; Hosier and Newton, 1958; Kibrick and Benirschke, 1958) and almost all of the patients were infants and children. The pathological changes in the liver consisted of mild fatty metamorphosis and severe focal necrosis of parenchymal cells, with or without inflammatory cell infiltration. The viral aetiological agent was isolated from the faeces and various organs such as the heart, brain, spinal cord, liver, kidney, spleen and pancreas, but Coxsackie viral crystals have never been demonstrated in the infected tissues.

Experimentally induced Coxsackie B viral hepatitis in animals has been mentioned in some previous reports (Godman, Bunting and Milnick, 1952; Minkowitz and Berkovich, 1970; Pappenheimer *et al.*, 1950, 1951). For example, Minkowitz and Berkovich (1970) recently reported Coxsackie B_1 viral hepatitis in adult mice in which the severity of hepatic lesions was related to the sex of the animals, with a male predilection. However, thorough histological and electron microscopic studies of hepatic lesions produced by viruses have not been reported.

During studies of viral cardiomyopathy in this laboratory, it was found that mice infected with Coxsackie viruses also developed damage to other organs as well as the heart. Because newborn and suckling mice are very susceptible to Coxsackie B virus infection and because Coxsackie virus B_1 has been found to form

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crystals in some infected tissues (Tsui, Burch and Harb, 1972), we extended the study to the liver. Thus, the pathology of viral hepatitis was studied in three groups of mice of different ages infected with Coxsackie virus B_1. In spite of extensive damage to the liver by the virus, viral crystals were not found in the liver even though they were found in the pancreas of three of the same animals. This report is concerned with a description of the hepatic lesions observed by light and electron microscopy.

MATERIALS AND METHODS

The virus.—The Coxsackie virus B_1 used in this laboratory was a stock virus received from the Communicable Disease Center in 1959. It has been passed in KB cells and twice in monkey kidney cells. The second viral passage from monkey kidney cells was used in these experiments. It has a titre of 10^{-4} $TCID_{50}$. The control fluid used was virus-free monkey kidney cell culture fluid which had been stored at $-65°$ along with the virus culture fluid.

Mice.—A random breed strain of HaM/ICR mice was used in these experiments.

Inoculation of virus and collection of tissues.—Sixteen 1 to 2-day old newborn mice, twelve 14-day old suckling mice and eight 5-week old young adult mice (5 female and 3 male) were inoculated intraperitoneally with 0·1 ml–0·2 ml of the Coxsackie virus B_1 culture fluid. The newborn mice were killed 1–3 days after inoculation, the 14-day old mice were killed 1–4 days after inoculation, and the young adult mice were killed 1–8 days after viral inoculation. The livers were examined grossly, and representative specimens from liver, pancreas and other tissues were selected for histological and electron microscopic studies.

Control studies.=Ten 1–2-day old mice, 9 14-day old mice and 4 young adult mice were inoculated intraperitoneally with 0·1 ml and 0·2 ml of virus-free culture fluid. The animals were killed simultaneously with the virus inoculated animals of corresponding age groups, the livers were examined grossly and tissues were collected for light and electron microscopic examination.

Histological studies.—The liver and other tissues from 26 infected animals and 13 control mice were fixed immediately with 10% neutral formalin. They were then processed, embedded in paraffin and sectioned to 6 μm thickness. The sections were stained by routine haematoxylin and eosin method.

Electron microscopic studies.—Tissues from 6 newborn and four 14-day old mice inoculated with Coxsackie virus B_1 and from 5 new born and five 14-day old control mice were prepared for electron microscopic examination. The tissues were prefixed in 3% phosphate buffered glutaraldehyde for 2 hours. Post-fixation was in 1% phosphate buffered osmium tetroxide for 1·5 hours. Dehydration was achieved with a graded series of methanol. The tissues were embedded in epon 812, and thin sections of 40–60 nm were stained with uranyl acetate and lead citrate. A Siemens Elmiskop I electron microscope was used for observations.

RESULTS

Gross findings.—The livers of the infected newborn and 14-day old mice were swollen and pale yellow in appearance, whereas the livers of the infected young adult mice showed very little change from normal, and those of the control mice appeared normal.

Light microscopic findings.—The histological changes of the liver consisted of focal to widespread cellular degeneration and necrosis with mild to no inflammatory cell infiltration. The Küpffer cells were prominent and sinusoidal congestion was usually present.

The degeneration consisted of " ballooning " of the parenchymal cells. The " ballooned " cells sometimes showed very little stained cytoplasmic material. The cells and nuclei varied in size. Some of the degenerative cells showed margination of nuclear chromatin with the remaining central area being degenerated and eosinophilic stained (Fig. 1A).

Cell necrosis was displayed by vacuolated cytoplasm with partially karyolytic nuclei (Fig. 1B), by " ballooning " of the cytoplasm with densely stained eosinophilic or karyolytic nuclei, by eosinophilic stained dense or loose cytoplasm with pyknotic or karyorrhectic nuclei, or by dense eosinophilic round bodies with or without small pyknotic nuclei in the liver cell plate or sometimes in the sinusoids. Occasionally, there was only one isolated partially karyolytic nucleus without surrounding cytoplasm in the cell plate. These round bodies from individual liver cell necrosis are called Councilman's bodies in yellow fever disease or acidophilic bodies in infectious or serum hepatitis in man.

There was focal cell necrosis (Fig. 2A) and widespread cell necrosis (Fig. 2B); and scattered individual necrotic hepatic cells were seen, being fairly numerous in the newborn mice and fewer in the young adults than in the 14-day old mice.

Generally, the degenerative and necrotic lesions were noted in the livers of both newborn and 14-day old mice by 24 hours after inoculation, but were not observed in the young adult mice until 48 hours after inoculation. The lesions increased in severity with time during the first 3 days in each age group of mice. The lesions were extremely severe in the newborn mice by the second and third days after viral inoculation. All infected newborn mice died spontaneously by the third day. Cell regeneration, evidenced by increased mitotic figures of liver cells with mild pathological changes, was noted in the liver of the suckling mice 4 days after infection. The young adult mice killed 8 days after viral inoculation showed some mitotic figures but were without any pathological changes.

There was little difference in the severity of the hepatic lesions among the mice within each individual age group except that some of the young adult female mice had slightly more cellular damage than the young adult males. Haemopoietic tissues are normally present in the liver of the newborn mice. Some of the megakaryocytes in the liver of the virus infected mice also showed degenerative changes.

The livers of the control mice were all histologically normal.

Electron microscopic findings.—In the livers of the infected animals, parenchymal cell damage was widespread. The damage ranged from moderate to severe. Among the most common hepatic cellular lesions were dilated rough endoplasmic reticulum, reduced density of the cytoplasmic ground substance and abundant membrane-bound vesicles and vacuoles of various sizes. The nucleus frequently lost its usual oblong or round shape and the outer nuclear membrane was ballooned out (Fig. 3). The nuclear chromatin was condensed around the periphery of the cell; the mitochondria remained intact.

In some liver cells, the damage to the cytoplasm was focal (Fig. 4). The destroyed areas varied in size from small to large. The areas of degeneration contained vesicles and myelin bodies. Remnants and fragments of membranes were also present. In some liver cells, there were areas in which groups of vesicles and vacuoles were located (Fig. 3).

Where the damage was severe the cytoplasm was completely disrupted (Fig. 5). The rough endoplasmic reticulum was dilated and numerous vesicles had formed. The cytoplasmic membrane was completely ruptured, so that the cytoplasmic contents were extruded into the extracellular spaces.

The nuclei exhibited changes in that the chromatin was marginated (Fig. 3) or condensed. The condensed nuclei apparently represent the pyknotic nuclei noted with the light microscope.

Almost complete destruction of the liver cell was noticed occasionally, with areas in which bodies with a similar structural appearance to cytolysomes were found to almost fill the cell (Fig. 6). The Küpffer cells also showed remarkable changes; small or large destroyed areas noted as cytolysomes were found in the Küpffer cells (Fig. 7).

A thorough search of many sections of the liver of the infected mice failed to reveal any Coxsackie B_1 viral crystals in either the parenchymal cells or Küpffer cells, even though crystals were readily found in other tissues of the same animals (Tsui *et al.*, 1972). Realizing the small size of the electron microscopic sections, it is still possible that viral crystals were present in the liver but not observed in these small sections in spite of an exhaustive search for them.

The livers from 3 newborn control mice and one 14-day old control animal examined with the electron microscope showed some abnormal changes in the parenchymal cells. These changes consisted primarily of degeneration with loss of cytoplasmic ground substance and clumping of the nuclear chromatin with loss of nucleoplasm. The rough endoplasmic reticulum remained intact, as did the mitochondria. There were no cytolysomes, such as were found in the infected animals, nor were there any large or small vacuoles in the pathological cells. These abnormal cells were not numerous, but were scattered randomly about the hepatic parenchyma. There were no changes observed in the Küpffer cells or sinusoids of these control animals. The changes observed in the livers of the control mice did not resemble those found in the livers of the experimentally infected animals.

DISCUSSION

Viral hepatitis is produced by many viruses in man (Davidson, 1970; Huang, 1971; Stein, Fainaru and Stein, 1972; Zuckerman, 1970) as well as in experimental animals (Minkowitz and Berkovich, 1970; Bergold and Weibel, 1962; Givan and

EXPLANATION OF PLATES

FIG. 1.—Sections of the liver of mice infected with Coxsackie virus B_1. (A) Hepatic cells of a 3-day old mouse killed 2 days after inoculation are swollen and display margination of nuclear chromatin (arrows). Some of the nuclei show prominent nucleoli. (B) Hepatic cells are vacuolated with karyolytic nuclei (arrows) in a 16-day old mouse killed 2 days after inoculation. H. and E. × 450.

FIG. 2.—Liver sections of 3-day old mice killed 2 days after inoculation with Coxsackie virus B_1. (A) Focal hepatic cell necrosis. H. and E. × 290. (B) Widespread liver cell necrosis. H. and E. × 60.

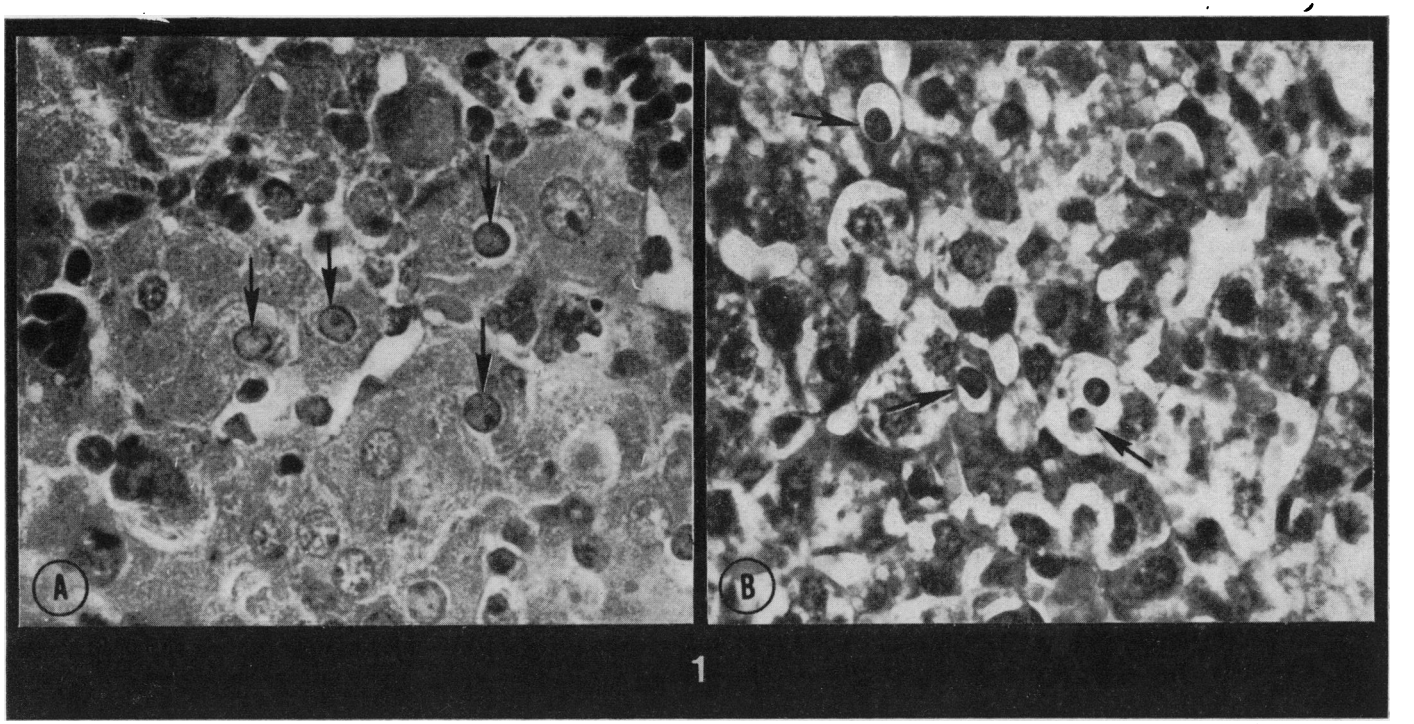
FIG. 3.—Electron micrograph of a hepatic cell from a newborn mouse killed one day after inoculation with Coxsackie virus B_1. The cytoplasmic ground substance is less dense than in the adjacent cell. There are abundant large vacuoles (V) in the cytoplasm. The rough endoplasmic reticulum is dilated. The nucleus is distorted with the nuclear chromatin condensed at the periphery. The outer nuclear membrane is ballooned (arrows). × 10,100.

FIG. 4.—Hepatic cell from a 14-day old mouse killed one day after viral inoculation. Note the large area of necrosis within the hepatic cell. Fragments of cell organelles are evident. Myelin-like bodies are present. The surrounding cytoplasm appears essentially normal. × 18,700.

FIG. 5.—Hepatic cell from a newborn mouse killed one day after viral inoculation in which the cellular architecture is completely disrupted. The ground substance is lacking in some areas. The cellular organization is lost. The rough endoplasmic reticulum is fragmented and dilated. × 18,700.

FIG. 6.—Hepatic cell from a newborn mouse killed 2 days after viral inoculation, showing a large cytolysome which almost completely fills the cytoplasm. × 15,600.

FIG. 7.—Küpffer cell from a newborn mouse killed 2 days after viral inoculation. Note the two cytolysomes (arrows) on either side of the nucleus (N); they indicate necrosis of the cell. Fragments and remnants of cellular organelles can be seen within these bodies. × 16,800.



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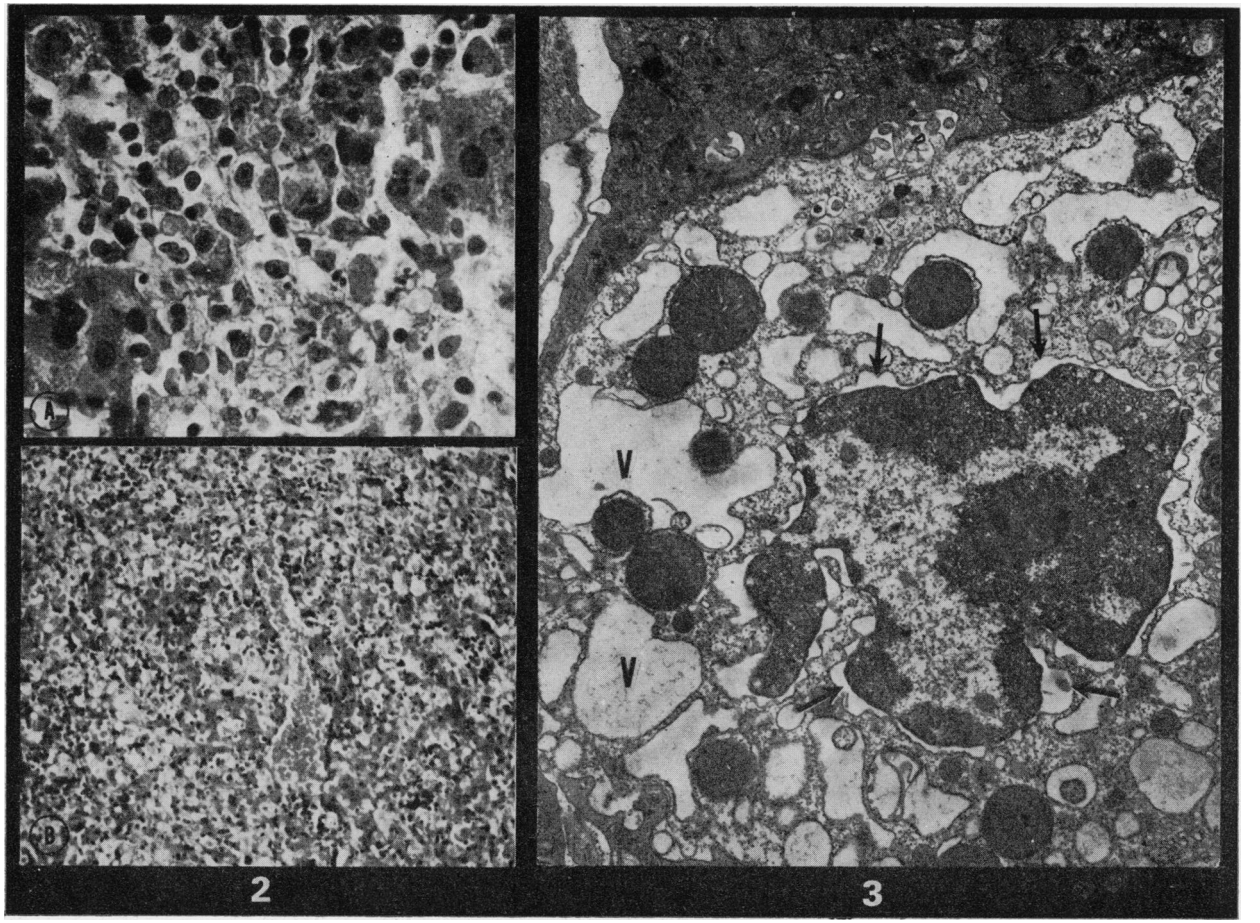

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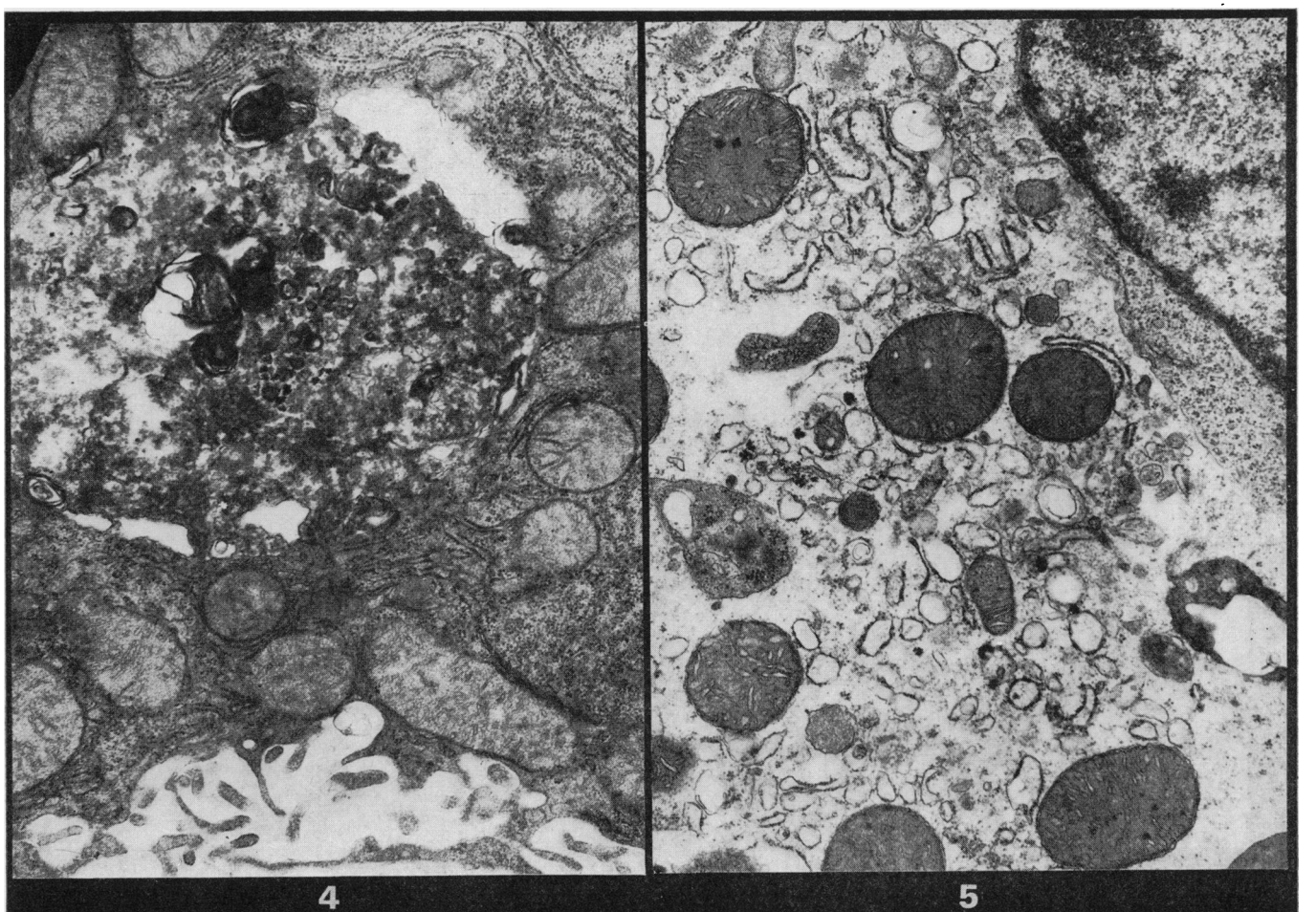

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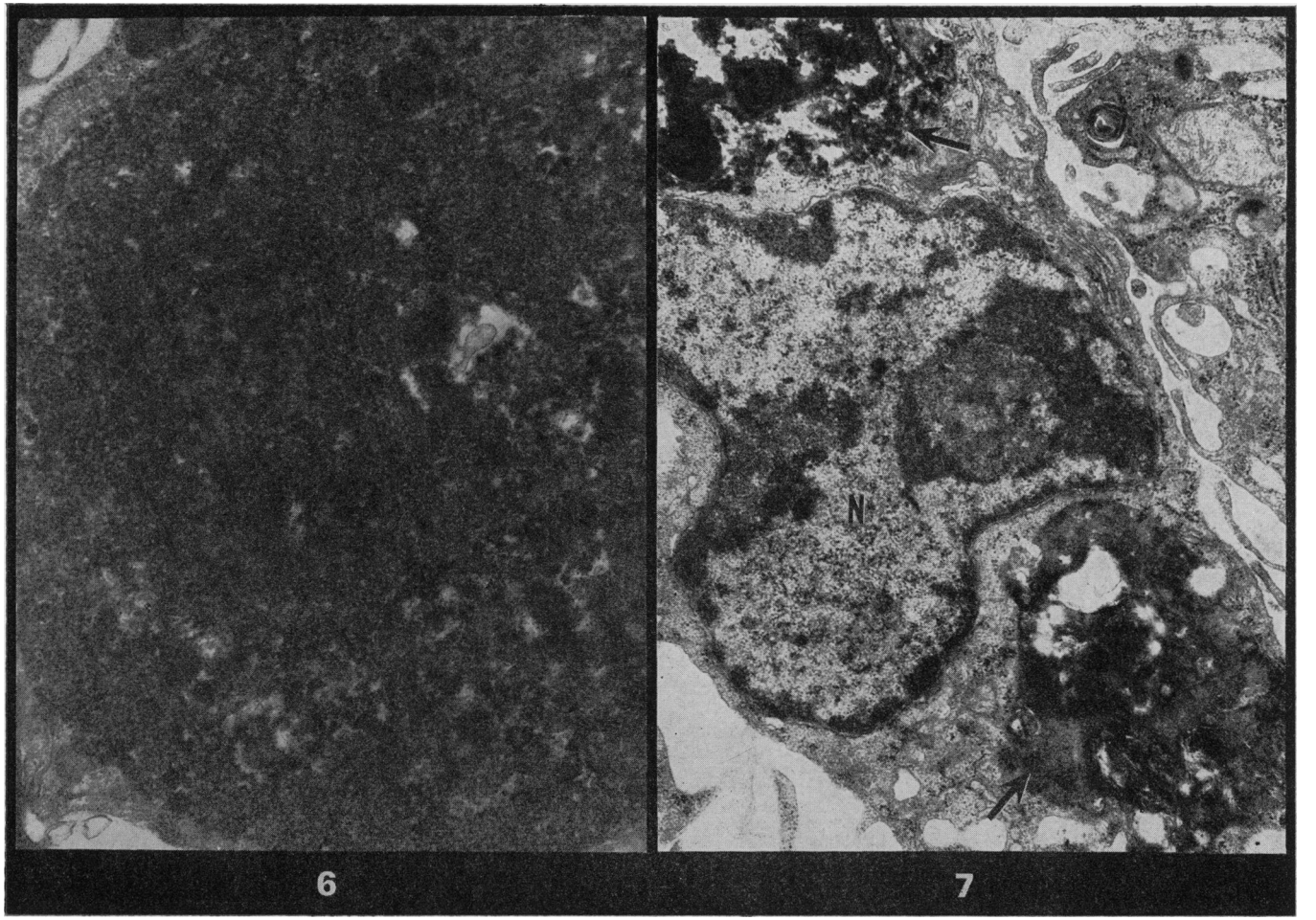
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Jézéquel, 1969; Miyai, Slusser and Ruebner, 1963; Radwanski, 1969; Svoboda *et al.*, 1962). The infectious and serum hepatitides have been responsible for most of the clinical cases of hepatitis. These hepatic diseases are believed to be due to viral infection, and the findings of Australia and SH antigens in patients with viral hepatitis are encouraging (Zuckerman, 1970). Furthermore, viral particles considered to be Australia antigen were demonstrated recently in liver biopsies of patients with viral hepatitis (Huang, 1971; Stein *et al.*, 1972). Hepatitis in man produced by Coxsackie B viruses (one of the most infectious groups of viruses for man) revealed marked pathological changes in the liver, but causative viral crystals were not observed. Although the Coxsackie viral crystals were not found in liver cells of the infected mice in our studies, these experiments show a causal relationship between the lesions in the liver and Coxsackie viral infection. The pathological changes observed in these mice are remarkably similar to those of infectious and serum hepatitides and hepatitis due to yellow fever in man. The failure to find viral crystals does not rule out the presence of virus in the liver. It is possible that the liver possesses certain specific structural and metabolic characteristics which inhibit or do not favour the development of Coxsackie viral crystal formation or even the development of readily recognizable viral particles. This seems particularly likely since three of these same mice which failed to reveal viral crystals in the liver even after exhaustive search and even in the presence of extensive pathological lesions did exhibit characteristic viral crystals in tissues of the pancreas. Furthermore, some areas of the liver cells showed changes similar to those noted in the pancreas where viral crystals were found. For example, areas were described in the cytoplasm of pancreatic cells in which groups of vesicles and vacuoles had formed and in which viral crystals were located (Tsui *et al.*, 1972). Also, in the liver and pancreatic cells of these same animals, areas were observed which we interpret as cytolysomes. Although viral crystals were found in these areas in the pancreas (Tsui *et al.*, 1972), we did not find viral crystals in similar areas in the liver. The liver cells with large cytolysomes seen with the electron microscope are considered to be acidophilic bodies noted with the light microscope. Acidophilic bodies have been shown to be dead liver cells (Klion and Schaffner, 1966).

The mechanisms responsible for the variety of cellular structural changes apparently produced by the virus are unknown.

In these experiments Coxsackie virus B_1 produced moderate to severe widespread hepatic lesions in both the newborn and 14-day old mice but only mild lesions in the young adult mice. The occasional focal areas of necrosis and diffuse cell necrosis were noted mainly in the newborn mice. Inflammatory cell infiltration was scanty. The Küpffer cells were prominent and showed severe ultrastructural damage.

The 14-day old mice showed much less cell damage by the fourth day after inoculation than during the earlier days of infection and also showed a considerable amount of cell regeneration, evidenced by a great deal of cell mitosis. The cellular degeneration and necrosis were very scanty in the young adult mice by the fourth day after inoculation and the livers recovered to normal with some evidence of mitosis by the eighth day after viral inoculation. The areas of focal necrosis may be expected to result in fibrosis, but this was not investigated. Obviously, since the mice were killed between 1 and 8 days after inoculation, the lesions observed represented only the early stages of liver cell injury. This type of experiment

extended over a long period of time would provide an excellent model for the study of the time course of pathological changes and repair of the liver in response to viral infections.

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