NON-FATAL MOUSE CYTOMEGALOVIRUS HEPATITIS

Combined Morphologic, Virologic and Immunologic Observations

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In mice infection with lethal doses of the murine cytomegalovirus (MCMV) results in a rapidly fatal disease with inclusions and focal necrosis in the liver as well as other viscera.[1,2] This report deals with an acute and non-fatal hepatitis resulting from infection of young adult mice with sublethal doses of MCMV. Data on virus production, interferon synthesis and sequential histologic changes in the livers of infected mice are reported as well as effects of x-irradiation on the course of the disease. These studies have relevance to human cytomegalic inclusion disease now that the human strain of cytomegalovirus has been implicated as a cause of liver disease in man.[3-7]

Material and Methods

Mice. Seven-week-old (24 to 28 gm) ICR/HA female mice free of naturally occurring cytomegalovirus infection were used exclusively. They were maintained on Purina® Chow with veterinary Terramycin® added to the drinking water.

Murine Cytomegalovirus. The Smith strain which has been serially propagated through ICR/HA mice was used in all experiments.

Experimental Infection of Mice. Suspensions of virus were prepared from the submaxillary glands of mice infected 1 month previously. Glands were aseptically removed from 3 mice, homogenized in 10 ml cold Hanks salt solution and diluted in Hanks. Virus suspension (0.3 ml) was injected intraperitoneally (IP) into mice. Three doses of virus were used: undiluted virus suspension, and virus suspensions diluted 10^{-2} and 10^{-3}.

Tissue Cultures. Primary monolayers of mouse embryonic fibroblasts prepared from ICR/HA mice were grown in 60 mm Petri dishes using Eagle's medium with 5 per cent calf serum. "L" cells were serially cultivated in 1-liter bottles in the same medium. They were trypsinized and seeded into Petri dishes for interferon assay.

Virus Assay. Virus was assayed by the plaque method.[8]

Neutralization Test. Neutralizing antibody in the sera of infected mice was measured by the plaque-reduction neutralization test.[9]

Interferon Assay. Interferon in livers of infected mice was assayed in "L" cells using encephalomyocarditis (EMC) as the challenge virus.[10] Livers removed from 2 mice were homogenized together in 10 ml cold Hanks solution for several minutes using a hand operated homogenizer. After 20 minutes centrifigation at 2,000 rpm, a sample was taken from the supernatant for virus assay and the remainder of the supernatant was dialyzed against 25 volumes NaCl-HCl buffer, pH 2.0, for 24 hours

Supported by the Otho S. A. Sprague Memorial Institute, the Illinois Division of the American Cancer Society and the National Institutes of Health, 2G-129, FR 05477.

Accepted for publication, May 27, 1966.

* Special Postdoctoral Research Fellow, National Cancer Institute, 1-F3-CA-30, 434-01.

at 4° C, then against Hanks solution for 24 hours. Next, the dialyzed supernatant was re-centrifuged as before, diluted with equal volumes of Eagle's medium containing twice the concentration of amino acids and vitamins, and assayed for interferon by titering EMC after 24 hours growth in "L" cells using a low input of infection.[10]

Experimental Procedure. Mice were given IP inoculation through the anterior abdominal wall with 0.3 ml virus suspension. The day after inoculation was considered as day 1. On subsequent days 2 mice were sacrificed, their livers excised and a portion fixed in 10 per cent formalin for histologic study. For virus and interferon studies, the rest of the liver was then weighed and homogenized as described in the preceding paragraph. Control mice were inoculated with 0.3 ml salivary gland homogenate prepared from non-infected mice of the same strain. Thirty-six to 54 mice were used in each experiment; 16 to 36 were given injections of MCMV (8 to 18 with each virus dilution when 2 dilutions were used in a single experiment) and the rest served as controls. Virus, inclusions and interferon appeared only in the livers of mice inoculated with MCMV.

In some experiments, mice were given a single dose of 450 r whole-body x-ray irradiation immediately before infection. In each experiment 4 groups of 9 to 12 mice were used; one was infected only, the second irradiated and infected, a third was a control only and the fourth a control irradiated group. A Westinghouse x-ray machine operating at 200 kv and 18 ma was used for radiation. The machine delivered 74 r per minute at 50 cm in air.

Experimental Results

Effect of Lethal Doses

General Observations. Intraperitoneal injections of undiluted virus suspensions were uniformly fatal for ICR/HA mice. Two or 3 days after inoculation mice first exhibited signs of infection: decreased activity, ruffling of fur, loss of weight and appetite, and hunching of back which progressed until death usually on days 3 to 6. Histologic studies revealed inclusions and focal necrosis in the liver consistent with reports of others.[1,2]

Effect of Sublethal Doses

General Observations. With 10^{-2} dilution, mice showed mild transient signs of infection 3 to 5 days after inoculation. These signs were similar to but much less severe than those observed in mice given undiluted virus. In 10 per cent or fewer of the infected animals, the signs progressed and the mice succumbed, usually on days 6 or 7. With 10^{-3} virus dilution the mice showed no signs of infection and there was no mortality. Regardless of the dilution of virus injected, mice became chronically infected as evidenced by persistent inclusions and virus in their salivary glands.

Lesions in the Liver. Mice were sacrificed for histologic study on days 1 to 4, 6, 10, 14, and at 4 and 8 weeks after infection. In control mice, occasional foci of mononuclear inflammatory cells were distributed at random throughout the parenchyma. They were widely scattered, less than 1 focus per low-power field, and were present also in other mice not used

for these experiments. No lesions attributable to cytomegalovirus or another virus could be recognized in the livers or other viscera in control mice.

In infected mice, MCMV produced a focal hepatitis with the number of lesions proportional to the dilution of virus injected. In some experiments with 10^{-3} dilution, inclusions or lesions were not observed in every section of liver. Intranuclear inclusions first appeared 30 to 40 hours after infection within hepatic parenchymal cells in the subcapsular region and the periportal zones (Fig. 1). With the development of cytomegaly, infected cells were ejected into the sinusoid leaving the hepatic cord with an irregular outline (Figs. 2 and 3). With extrusion from the cords, local Kupffer cells proliferated (Figs. 4 and 5) and formed a cuff about infected cells. Neutrophils and lymphocytes appeared and the infected cells became sequestered within the sinusoids (Fig. 6) and underwent degeneration. The nuclear membrane wrinkled and collapsed obliterating the halo around the inclusion. Almost simultaneously, the inclusion body became irregular, less well defined, and disappeared (Figs. 7 to 9). Occasionally, the inclusion disappeared first, and the nuclear membrane was reduced to a small ring with an eosinophilic center. The entire cell became smaller (Fig. 10), homogeneous, and acidophilic and resembled the Councilman body of yellow fever (Fig. 11). Finally, acidophilic bodies fragmented and appeared to be engulfed by surrounding Kupffer cells. It took less than 36 hours for an infected cell to become extruded into the sinusoid and degenerate into an acidophilic body.

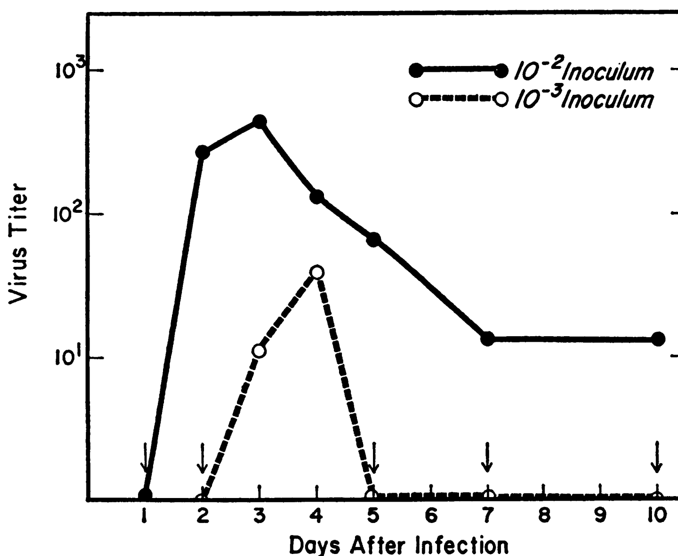
Inclusions were not seen in bile duct epithelium, though inflammatory cells frequently were visible within portal triads. There was no evidence of bile stasis.

At 72 hours, new inclusions were visible at the peripheral margins of only a small minority of foci, and these were reduced to acidophilic bodies. The appearance of new distant foci was rare and did not occur after the fourth day. By then, lesions had frequently expanded to a width of 4 to 5 hepatic cords, though they varied considerably in size, and were composed of Kupffer cells, lymphocytes, neutrophils and occasionally eosinophilic debris (Fig. 12). Adjacent hepatic cords were compressed and distorted, but at a distance from the lesion the parenchyma appeared normal. The number of lesions per section of liver varied slightly from one experiment to another, even with the same dilution of virus, but were always greater in mice infected with 10^{-2} dilution.

Intact intranuclear inclusions and acidophilic bodies were rare and not seen after the fifth day. From the sixth to the tenth day, lesions regressed, and, by day 14, only a few lymphocytes and Kupffer cells remained. After day 5, the number of mitotic figures in the liver cells of

infected mice was significantly greater than in non-infected controls. After 4 weeks, no residual inflammation was seen. At 2 months, livers appeared architecturally normal with no progressive fibrosis or cirrhosis.

Virologic Studies. MCMV titers in the livers of infected mice are shown in Text-figure 1. Peak titers occurred on days 2 to 4 and were



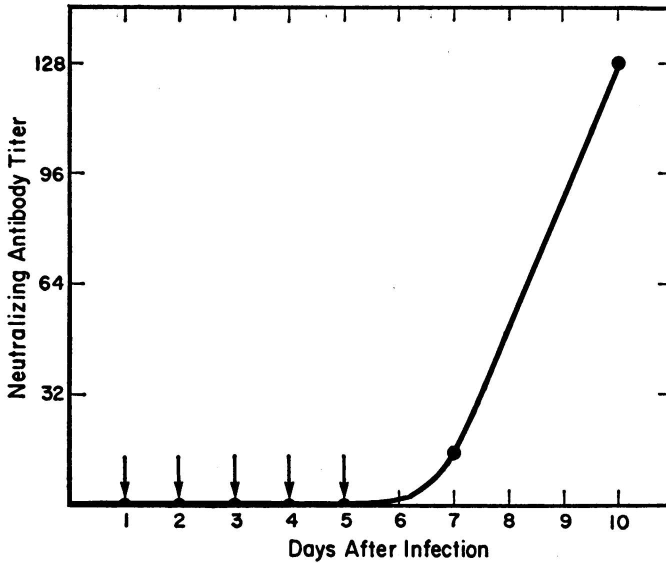
TEXT-FIG. 1. Virus production in the livers of 7-week-old mice after IP inoculation with 2 different dilutions of virus. The virus titer is expressed as the $\log_{10}$ plaque-forming units in 0.2 ml per gm of liver as a 20 per cent homogenate.

proportional to the dilution of the virus injected. Titers correlated with the relative number of inclusions only on days 2 and 3. Thereafter, inclusions rapidly disappeared though, with the 10^{-2} inoculation, infectious virus persisted in the liver during the first 10 days after infection. Similar virologic data have been reported in weanling Swiss mice infected IP with MCMV.[11]

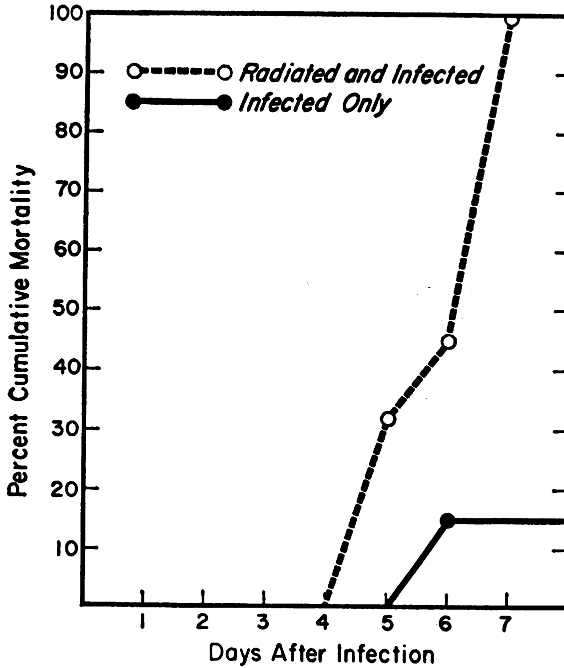
Appearance of MCMV Neutralizing Antibody. Serum neutralizing antibody first appeared on the seventh day (Text-fig. 2) after which the titer greatly increased.

Result of X-irradiation. The deleterious effect of 450 r whole-body irradiation on survival after MCMV infection is shown in Text-figure 3.

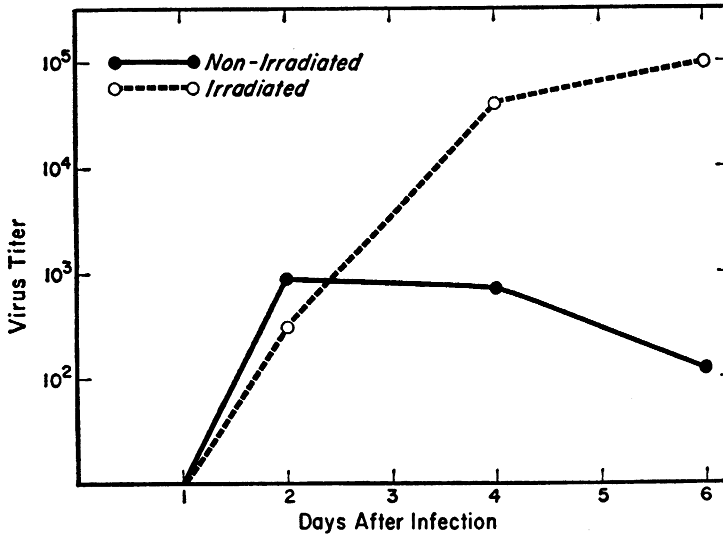
Histologic changes in liver cells in control irradiated mice were most marked on days 6 to 8. They consisted of nuclear hypertrophy, chromatin clumping and vacuolization of the cytoplasm. Kupffer cells were swollen and their nuclei hyperchromatic. In infected mice x-irradiation resulted in progression of the disease with expanding lesions often extending along the cords, necrosis and florid inclusions (Figs. 13 to 16).



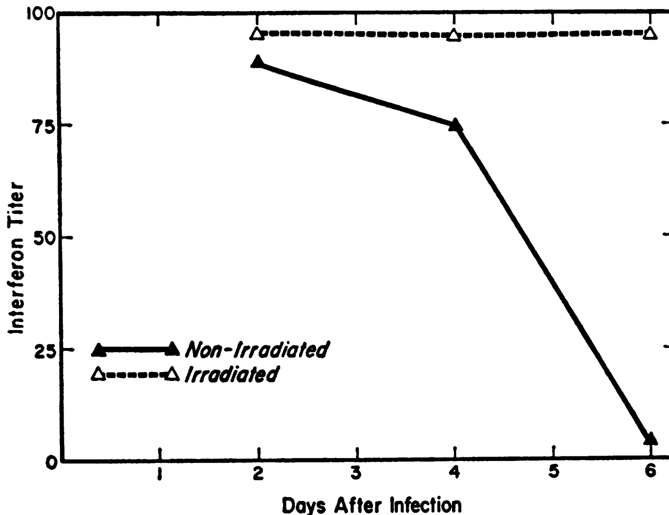
TEXT-FIG. 2. Appearance of neutralizing antibody in the serum of MCMV infected mice as measured by the plaque reduction neutralization test.[9] Mice were infected with 10^{-3} virus dilution. Antibody titer is expressed as the reciprocal of the serum dilution reducing the plaque count 50 per cent.



TEXT-FIG. 3. Effect of 450 r whole-body x-irradiation on survival after MCMV infection. Mice were inoculated IP with 10^{-3} dilution of virus immediately after irradiation. None of the control irradiated mice died during the time of the experiment (data not shown in the Text-figure.)



TEXT-FIG. 4. Virus production in the livers of x-irradiated and non-irradiated mice on days 2, 4 and 6 after infection with 10^{-2} dilution of virus. The virus titer is expressed as in Text-figure 1.



TEXT-FIG. 5. Interferon production in the livers of x-irradiated and non-irradiated infected mice on days 2, 4 and 6 after inoculation with 10^{-2} dilution of virus. Interferon titer is expressed by the formula:

$$\text{Titer} = 100(1-x)$$

where x represents the ratio of EMC virus titers obtained with homogenates made from the livers of infected mice to the EMC titers obtained with homogenates from the livers of control mice per gm of tissue.

There was no Kupffer cell or inflammatory cell response and acidophilic bodies did not develop. Examination of other organs indicated wide viral dissemination. Inclusions were present notably in the spleen, lymph nodes and kidneys.

On day 2, virus titers and interferon titers were almost similar in the irradiated and non-irradiated infected mice. After that, however, in the irradiated mice, virus titers increased and interferon was continuously produced. (Text-figs. 4 and 5). Neutralizing antibody was not measured in the irradiated mice since most animals expired from MCMV infection before antibody appeared in non-irradiated mice. Neither virus nor interferon was detected in the livers of mice from the control groups.

Result of Reinfection. Chronically infected mice at 6 weeks post-inoculation and mice not previously exposed to MCMV were given undiluted virus by IP injection. All mice were approximately 12 weeks of age and had been maintained under similar laboratory conditions. The chronically infected mice remained well and active, exhibiting no outward signs of infection. Histologically, only a few periportal mononuclear cells were noted at 4 days, but there were no inclusions. Mice not previously exposed to MCMV all died and showed the characteristic changes of lethal infection with numerous inclusions and liver necrosis.

Discussion

Murine cytomegalovirus in sublethal doses can produce a focal acute hepatitis in mice without residual cirrhosis or permanent liver damage. This is in contrast to lethal doses which characteristically produce necrosis and spreading foci of infection.[1,2] That cytomegalovirus caused the hepatitis was evidenced by the existence of specific inclusions as well as the demonstration of virus replication in the liver during the time of hepatic damage. The distribution of lesions in the liver most likely was the result of the IP route of injection. Subcapsular foci reflected virus penetrating the capsule and the periportal lesions indicated the entrance of virus via portal veins. The time of appearance and distribution of lesions were considered characteristic for IP injection. We do not know, however, if a similar distribution would follow other routes of inoculation or occur in much younger mice.

Kupffer cell proliferation is not limited to MCMV infection. It occurs in other viral diseases of the liver and during hepatocellular necrosis in man and in laboratory animals.[12,13] Using the inclusion as a marker, it was demonstrated that Kupffer cells proliferated and surrounded infected hepatic cells which degenerated into acidophilic bodies. This coincided with a decrease in virus and interferon production in the liver and preceded the appearance of neutralizing antibody by several days. In irradiated mice, the Kupffer cell as well as the inflammatory cell response was abolished, and there was continued viral replication, interferon production and expanding foci of necrosis with florid inclusions. Acidophilic bodies were not found in the irradiated mice. Our data suggest, therefore,

that Kupffer cells along with neutrophils and lymphocytes play a primary role in localizing MCMV infection in the liver and may be responsible for transforming infected hepatocytes into acidophilic bodies.

One useful fact emerging from these studies is that x-irradiation at doses sufficient to abolish the inflammatory response in the liver did not inhibit interferon production. Thus, it may now become possible to define more precisely the roles of interferon and inflammation during virus infection of the liver (and perhaps in other organs as well).

It should be emphasized that the material extracted from livers of infected mice was not characterized completely, though it fulfilled many properties of interferon.[14] It was non-dialyzable, stable at pH 2.0, active against a heterologous virus, not sedimented at $100{,}000 \times g$ and was present only in infected animals. The interferon titer correlated with synthesis of MCMV in the liver and with the relative number of inclusions. We could neither isolate a second virus in "L" cells or mouse embryo cultures nor observe cytopathic changes attributable to another virus in non-irradiated or irradiated mice. Furthermore, since interferon was not found in control irradiated animals, it seems unlikely that x-irradiation activated a latent virus in MCMV infected mice which could have been responsible for stimulating interferon production. For these reasons we conclude that interferon was produced in the liver, most likely by hepatocytes, secondary to MCMV infection.

These studies have relevance to human cytomegalic inclusion disease now that hepatomegaly, abnormal liver function tests and cirrhosis have been shown to be possible sequelae to human cytomegalovirus infection.[3–7] In view of the biologic similarities between the human and murine cytomegalovirus,[15] it is suggested that the human strain may be capable of producing a hepatitis in man similar to the MCMV hepatitis in mice.

Summary

Sublethal doses of the murine cytomegalovirus, when injected intraperitoneally into mice, produced a focal hepatitis with virus replication, interferon production and intranuclear inclusions in hepatic cells. Infected cells were extruded into the sinusoids, became surrounded by proliferating Kupffer cells and leukocytes and degenerated into acidophilic bodies in less than 36 hours. Lesions remained focal and regressed rapidly. The severity of the hepatitis and virus production correlated with the dilution of the virus injected. Mice recovered though they became chronically infected. No progressive hepatic fibrosis or cirrhosis was evident up to 2 months after infection.

Viral neutralizing antibody did not appear in the serum until 7 days

after infection. Chronically infected mice, however, were immune and did not develop a hepatitis after re-inoculation with MCMV.

A single dose of 450 r whole-body x-irradiation given immediately prior to infection abolished the Kupffer and inflammatory cell response in the liver and resulted in progression of the disease with expanding foci of necrosis, florid inclusions and death. Virus titers increased progressively, and interferon was continuously produced in livers of irradiated mice.

The data suggest that Kupffer cells and perhaps lymphocytes and neutrophils play a role in localizing MCMV infection in the liver.

Human cytomegalovirus may produce a hepatitis in man similar to that induced by the murine cytomegalovirus in mice.

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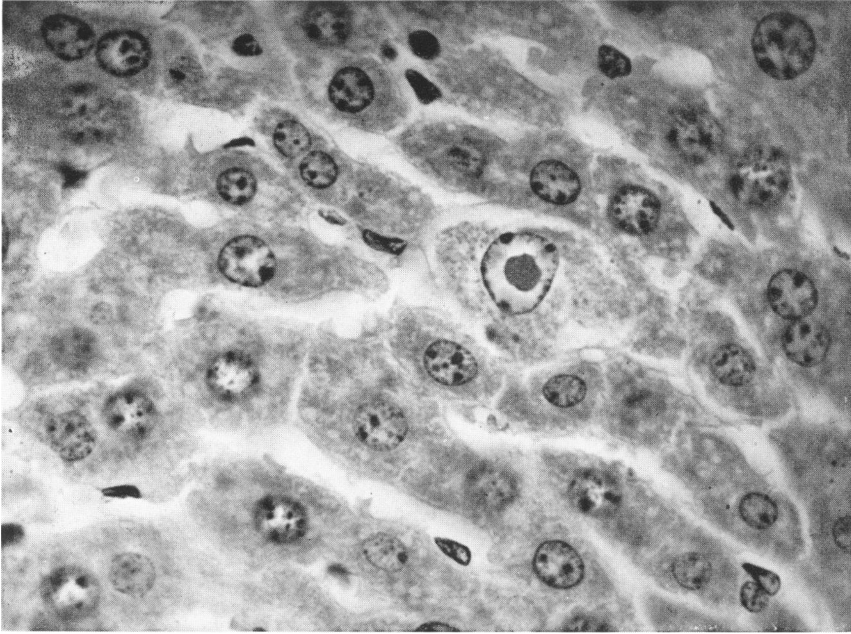
LEGENDS FOR FIGURES

Photomicrographs were made from sections stained with hematoxylin and eosin.

FIG. 1. An intranuclear inclusion is evident in an hepatic parenchymal cell 2 days after infection. × 725.

FIG. 2. An infected liver cell is in process of being extruded from the cord (plate) and is becoming surrounded by proliferating Kupffer cells. × 725.



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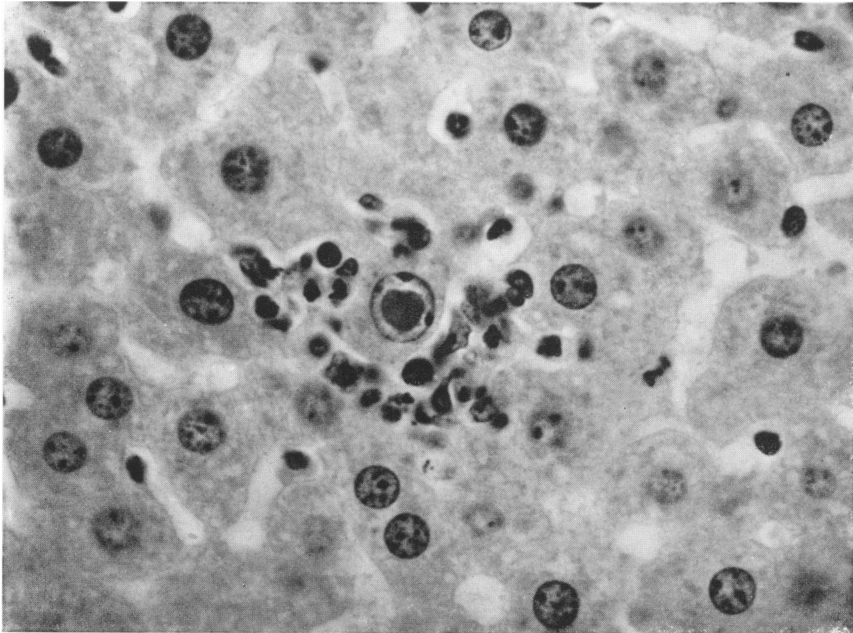

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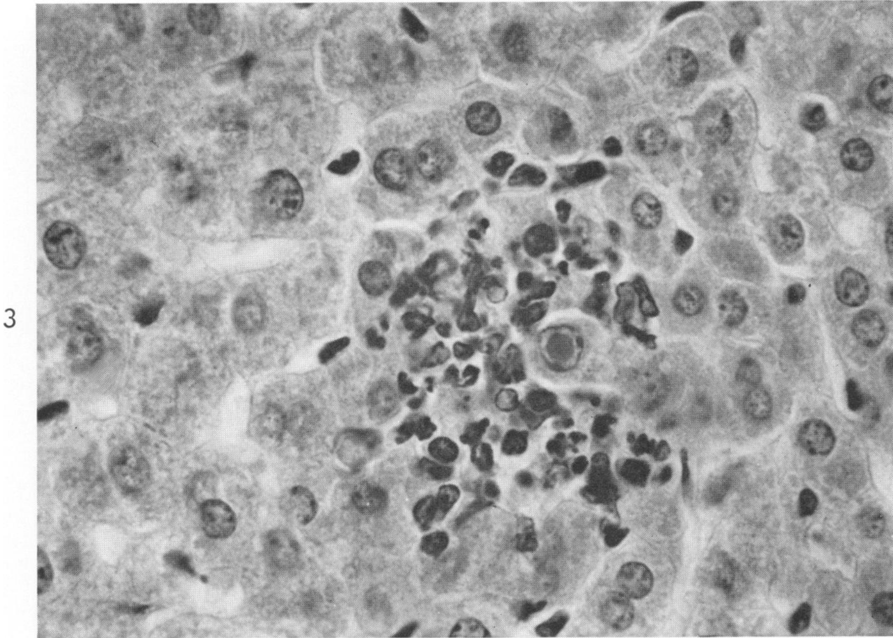

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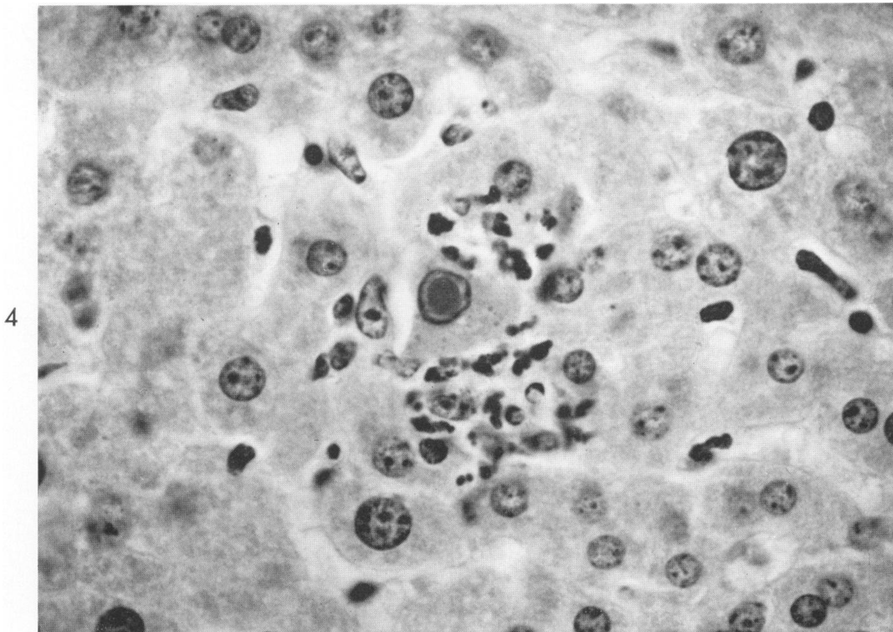
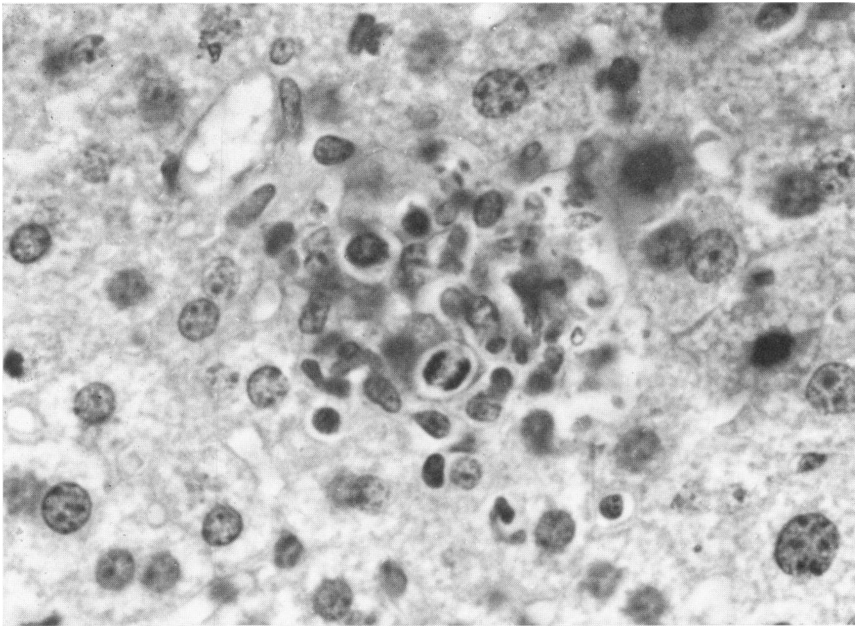


FIG. 3. An infected cell is becoming sequestered by Kupffer cells, lymphocytes and neutrophils as it emerges from the cord. Day 2. × 725.

FIG. 4. An infected liver cell lies in the sinusoid and is partly surrounded by several Kupffer and inflammatory cells. Two days after infection. × 725.



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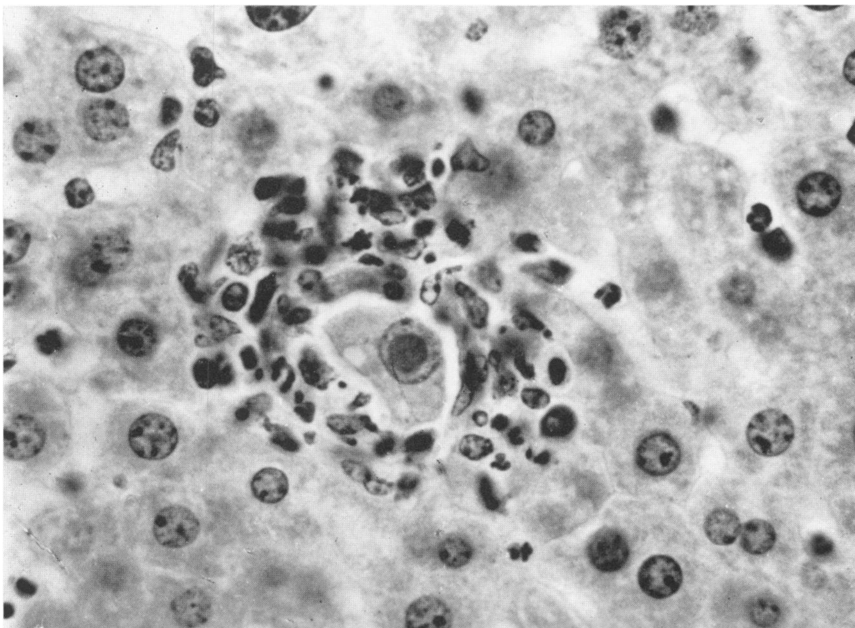

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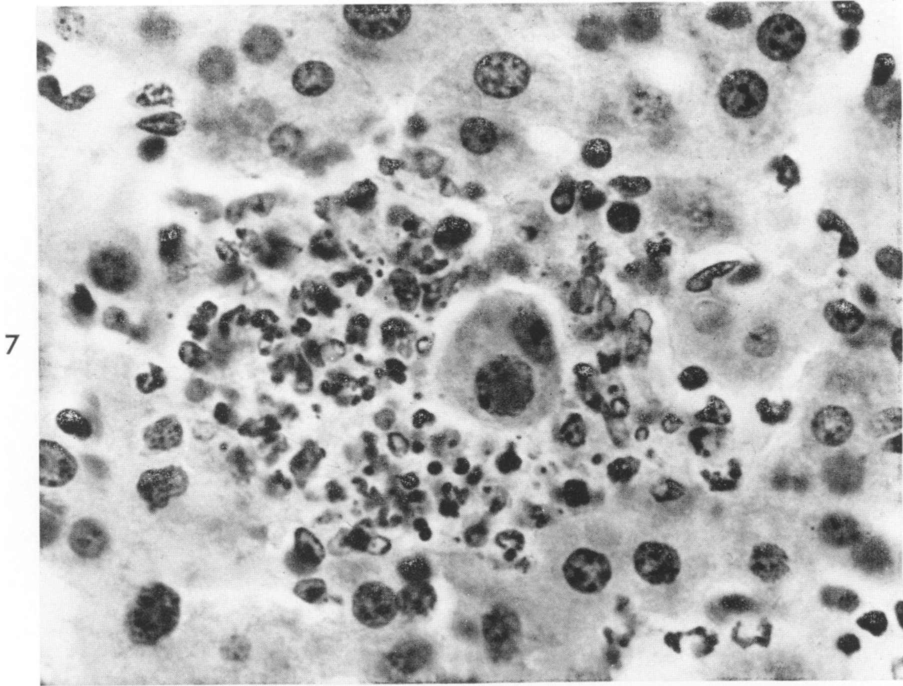
FIG. 5. In a small focus of inflammation a mitotic figure appears in a Kupffer cell. The inclusion is not visible in this plane of section. Day 2. × 860.

FIG. 6. A completely sequestered infected cell. Day 2. About 50 per cent of the inflammatory cells were considered to be Kupffer cells. × 725.

7



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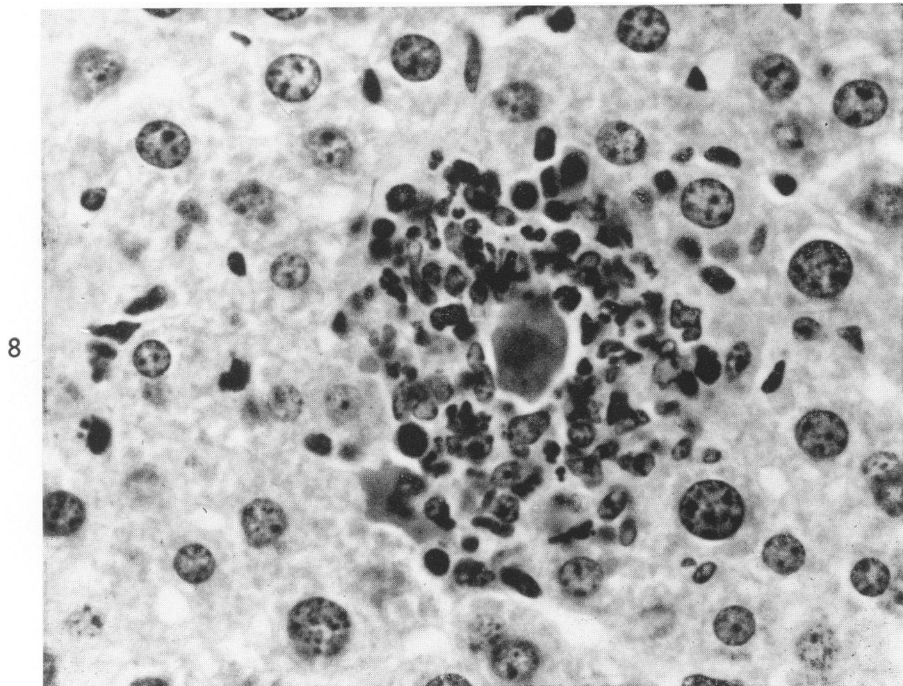
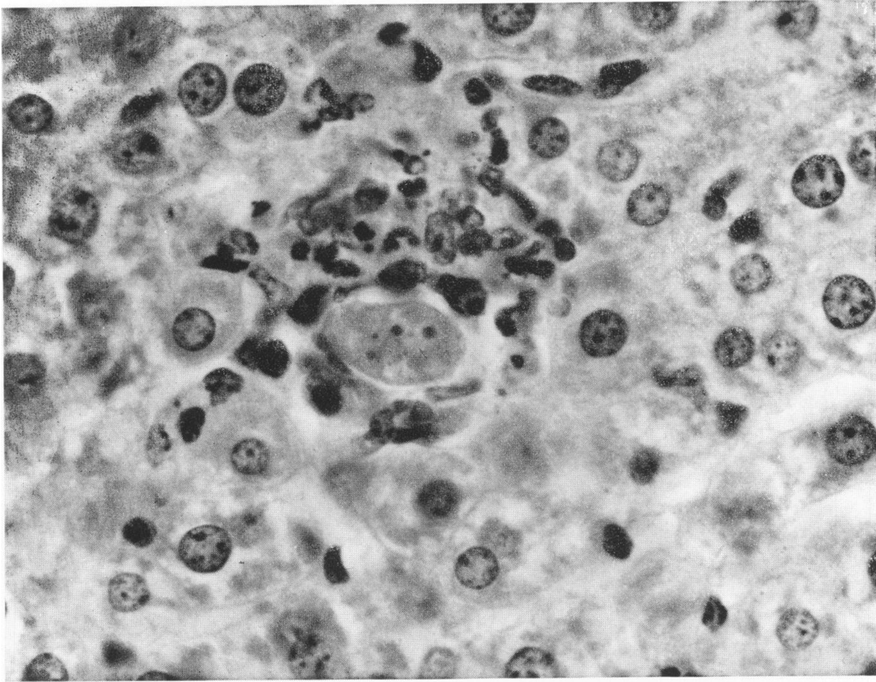


FIG. 7. A sequestered binucleated cell exhibits early stages of degeneration. The nuclear membrane is beginning to wrinkle and the inclusion to lose its sharp outline. Day 2. × 725.

FIG. 8. With further degeneration of an infected cell the nuclear membrane has almost disappeared. The inclusion is irregular, poorly defined and beginning to contract. It eventually shrinks and disappears. Day 2. × 725.



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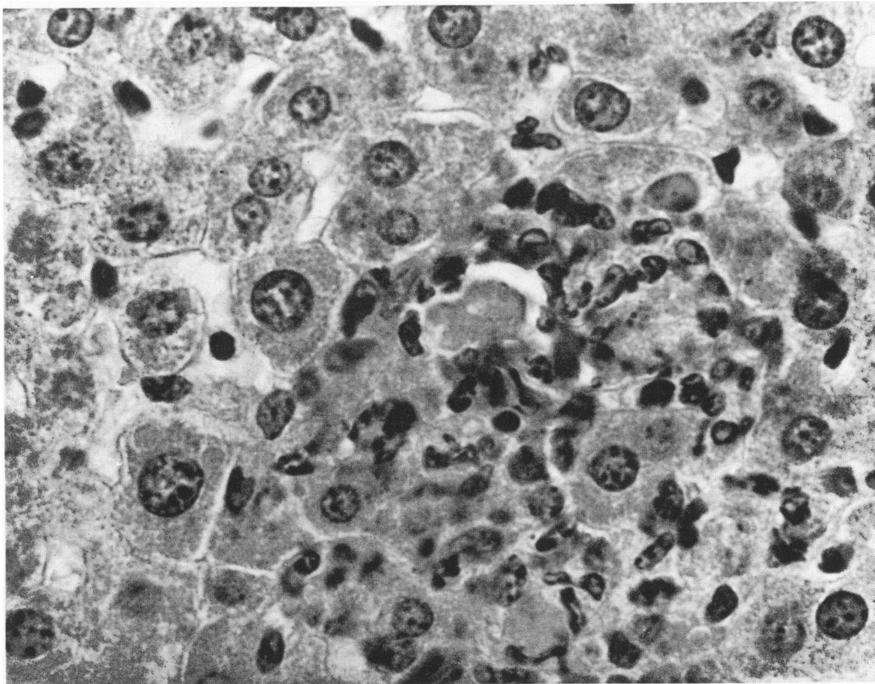

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FIG. 9. The nuclear membrane and inclusion have disappeared though nucleoli remain as small dark round bodies. Day 2. × 725.

FIG. 10. Complete loss of nuclear material has occurred. The cell is now considerably smaller and brightly eosinophilic. × 725.

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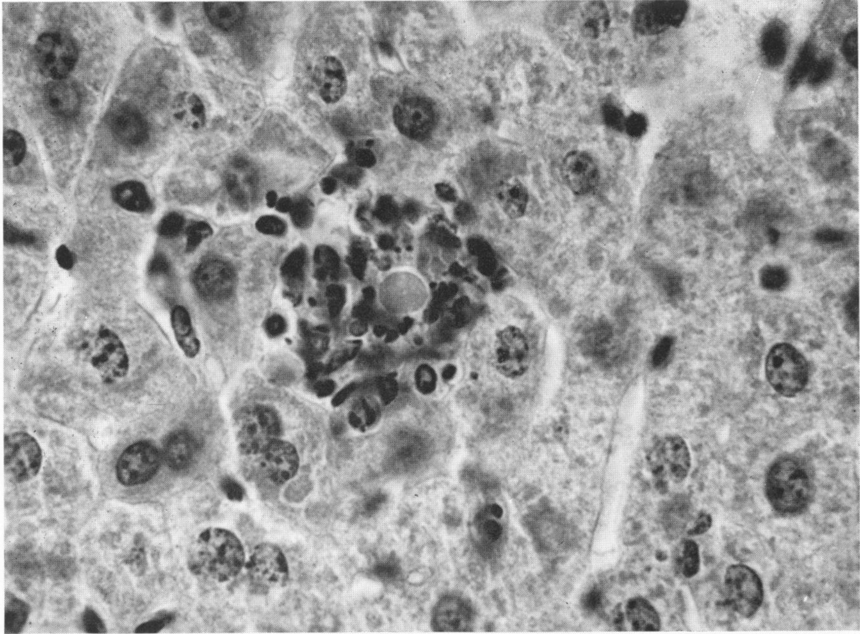

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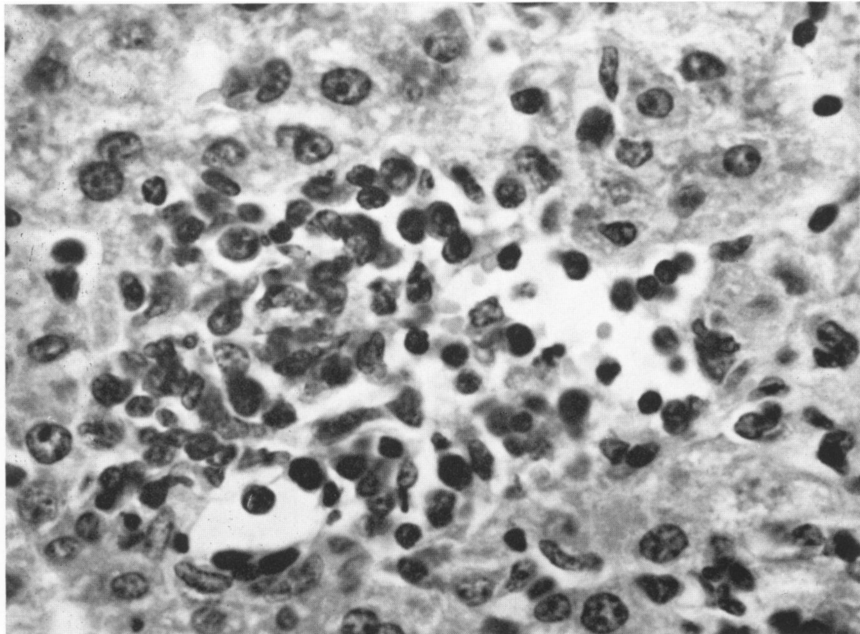
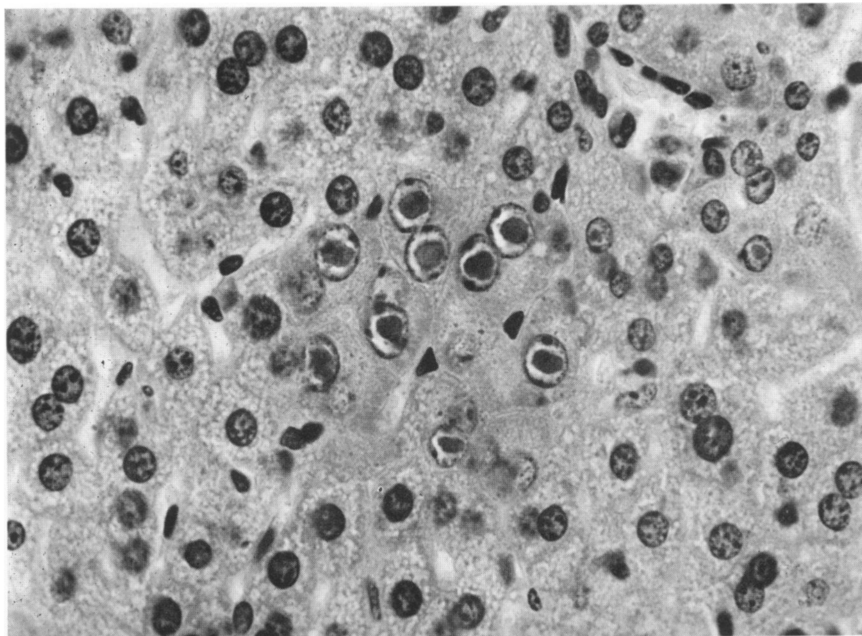


FIG. 11. An acidophilic body appears in the center of a cluster of inflammatory cells. Day 2. × 725.

FIG. 12. In a maximum lesion 3 days after infection the acidophilic body is no longer visible. The sinusoids are dilated and the hepatic cords are distorted by inflammatory cells. These lesions soon regress and by 2 weeks, only a few cells remain. × 725.



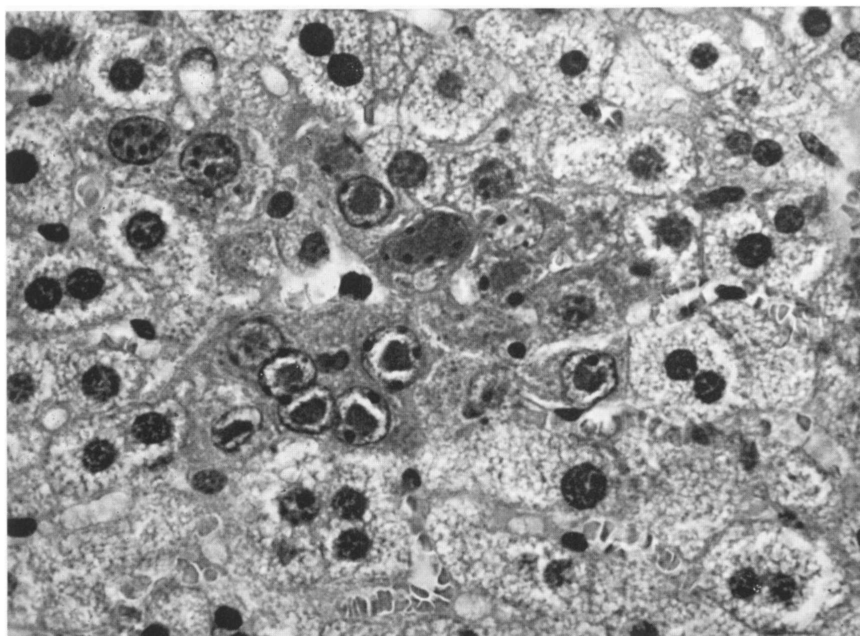
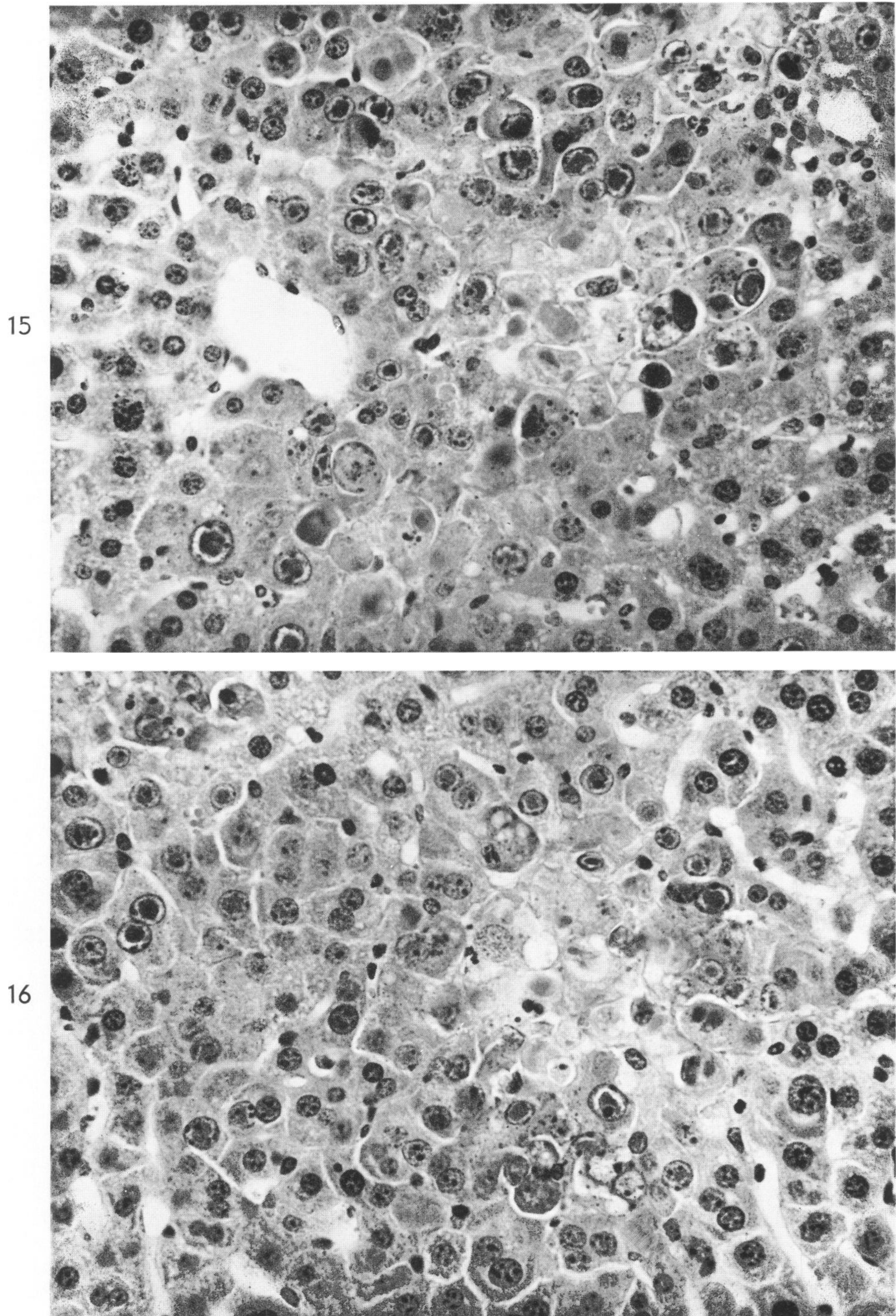


FIG. 13. Irradiated mouse 4 days after infection with MCMV. A spreading focus of infection in the liver lacks proliferating Kupffer cells, inflammatory cells and acidophilic bodies. × 725.

FIG. 14. Irradiated mouse 4 days after infection. Marked vacuolization of liver cells was observed in the irradiated animals. × 725.



FIGS. 15 and 16. Irradiated mice 6 days after infection. Large foci of infection contain many inclusions. There are central areas of coagulation-like necrosis where infected liver cells persist as shadowy outlines. No acidophilic bodies are apparent. × 560.