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EFFECT OF MOUSE HEPATITIS VIRUS INFECTION ON IRON RETENTION IN THE MOUSE LIVER

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Summary.—Increased iron uptake and iron-induced ferritin synthesis has been demonstrated in experimentally virus-infected cell cultures. However, this has not been observed in the intact animal. The results reported in this paper indicate that higher deposition of injected radioiron occurs in the livers of mice infected with MHV-3 virus compared with livers from uninfected animals. Administration of iron at different time intervals indicated that iron uptake correlates well with the degree of tissue injury in the livers of infected animals.

VIRUS-INDUCED cellular increases in iron uptake have been reported in a number of different systems. *In vitro*, an increase in iron uptake and ferritin synthesis were shown in L cells infected with Newcastle disease virus (Husain *et al.*, 1965), in Herpes simplex virus-infected HEp-2 cells (Lambert and Husain, 1973). *In vivo*, an increase in iron uptake was demonstrated in the spleen of mice infected with Friend leukaemia virus (Mirand *et al.*, 1961; Hankins *et al.*, 1973). More recently, an increase in serum iron levels was found to concur with persistent Australia antigen in humans (Sutnick *et al.*, 1974).

The difficulty in the study of human viral hepatitis and its possible relationship to iron storage disease is that it lacks an experimental model. Australia antigen is not known to infect common laboratory animals other than the simian host which requires special housing facilities.

Even though mouse hepatitis virus is not known to be related to human hepatitis virus, the fact remains that both viruses are involved in hepatic injury.

Therefore, it was reasoned that mouse hepatitis virus might provide a suitable model for an *in vivo* experiment involving iron-virus interaction.

MATERIALS AND METHODS

Mice.—In all experiments, weanling Swiss–Webster mice weighing 8–10 g were used.

Virus.—Murine hepatitis virus Strain 3 (MHV-3) Stock No. 8169/8 was originally obtained from Dr Michael J. Collins, Microbiological Associates, Bethesda, Maryland, U.S.A. The original stock virus at Microbiological Associates was prepared in tissue culture cell line NCTC 1469.

Propagation of stock virus.—Mice were inoculated intraperitoneally with 0·2 ml of a 1/10 dilution of viral suspension of the original stock virus in cold 1X Hanks' Balanced Salt Solution (HBSS). The infected mice were observed for 10 consecutive days. Mice that died within the first two days were discarded as non-specific. Moribund mice were killed by cervical dislocation, and infected livers which showed white plaques were pooled. Pooled infected livers were minced and homogenized in a Ten Broeck glass homogenizer, diluted to make a 10% (w/v) suspension with cold 1X HBSS.

The homogenate was centrifuged in a refrigeratred centrifuge at 205 *g* for 15 min. Supernatant was transferred and recentrifuged at 12,000 *g* for 30 min. Antibiotics were added to the cell-free supernatant of infected liver

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homogenate at a concentration of 250 units of penicillin G plus 250 μg of streptomycin/ml. The stock viral suspension from liver homogenate at the fourth passage was divided into several 2 ml amounts, and kept at $-76°$ until used. Infectivity titre of the stock viral suspension was determined by the method of Reed and Muench (1938), and was found to be $10^{-2.75}$.

Experimental virus infection of mice.—Mice were infected with 10 LD_{50} of the titrated stock virus in 0·2 ml of cold HBSS.

Iron solution: stock ferrous sulphate solution (5 mg/ml).—Ferrous sulphate (crystals), $FeSO_4 \cdot 7H_2O$, mol. wt 278·02 (Lot No. 710921, Fisher Scientific Company, Fair Lawn, New Jersey, U.S.A.) was weighed and dissolved with iron-free double-distilled water in a chemically clean volumetric flask to make a solution, divided into several small amounts and kept in a $-20°$ freezer until used.

Radioactive iron solution (^{59}Fe).—Solution of ^{59}Fe in the form of ferric chloride in 0·5M HCl, Lot No. 60021 (specific activity 12·5 mCi/mg) was purchased from New England Nuclear Corp., Boston, Massachusetts, U.S.A. Upon arrival, the radioiron was diluted 1/10 with 0·9% NaCl.

Radioiron assay.—A mixture containing 2 μCi of ^{59}Fe and 140 μg carrier iron as $FeSO_4$ contained in 0·5 ml of 0·9% NaCl was injected into fasting uninfected and infected mice. Earlier experiments indicated that this amount of radioiron and carrier iron was well tolerated by mice within the experimental period. For radioactive counts, whole liver was removed from sacrificed mice and washed 3 times in 10 ml of 0·9% NaCl. Three washes were usually sufficient to remove exogenous radioiron from the mouse liver.

The washed livers were subjected to radioisotope counts in a well-type Packard Auto-Gamma spectrometer Model 2002 (Packard Instrument Company, Inc., Downers Grove, Illinois, U.S.A.). Duplicate counts were obtained for each specimen. The average count per minute (ct/min) per organ was recorded. The liver was partially dried on a filter paper, weighed and ct/min/1 g of liver (wet weight) was computed. Counts were plotted *versus* time in hours.

Electron microscopic examination.—Mouse liver samples taken immediately after death were minced and fixed in 2·5% glutaraldehyde in 0·1M phosphate buffer pH 7·2. They were postfixed in 1% osmium tetraoxide in 0·1M phosphate buffer pH 7·2. They were washed repeatedly in distilled water, dehydrated through a graded series of ethanols and acetones, and embedded in Spurr's plastic. The plastic blocks were then trimmed and sectioned on a Sorvall Porter-Blum Microtome (Ivan Sorvall, Inc., Norwalk, Connecticut, U.S.A.) equipped with a glass knife, to an approximate thickness of 90 nm. The ultra-thin sections were mounted on 200 or 300 mesh uncoated copper grids, and subjected to stain with 1% uranyl acetate in 50% ethanol for 10 min, followed by 3 min of staining with lead acetate. The stained sections were examined and photographed on a Hitachi HU-11E electron microscope at 50 kV.

RESULTS

The amount of radioiron uptake in the livers of uninfected and infected mice

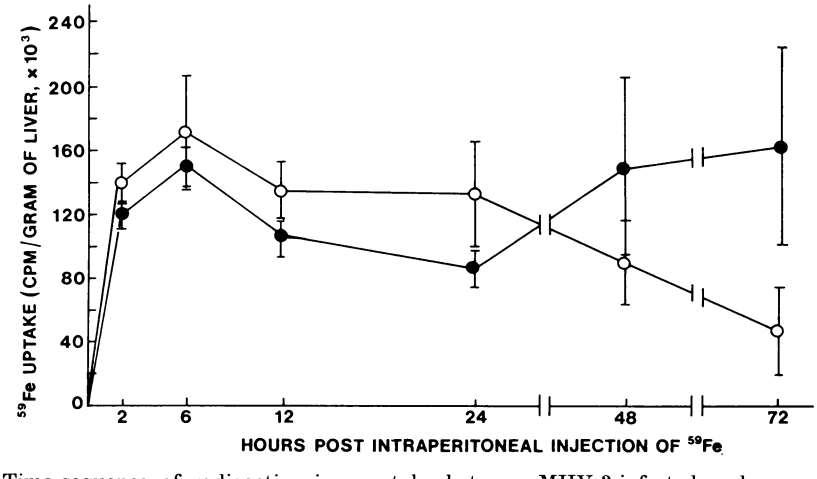


FIG. 1.—Time sequence of radioactive iron uptake between MHV-3-infected and normal mouse livers. The quantity of iron uptake was measured at 2, 6, 12, 24, 48 and 72 h after a single administration of iron into mice which were previously infected with the virus for 1 day. Uninfected livers (○), infected livers (●). Each point is the mean of 5 measurements ± standard error.

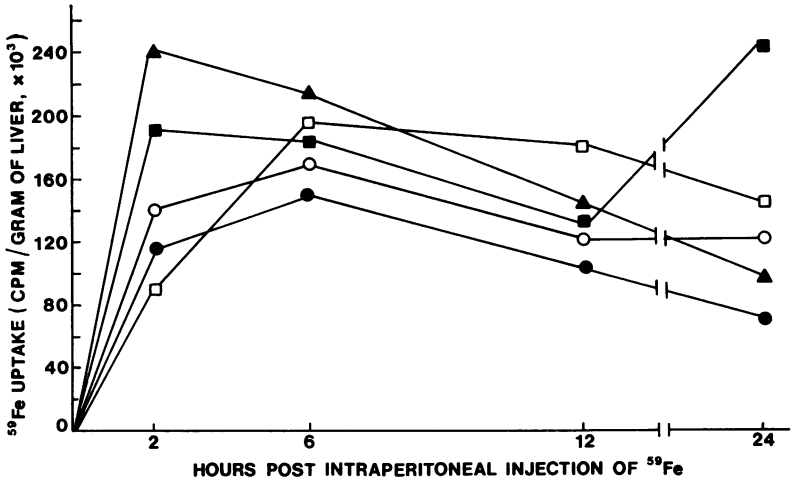


FIG. 2.—24 h course of radioactive iron uptake by MHV-3-infected mouse livers at various stages of infection. Uninfected (○), 1 day (●), 2 days (□), 3 days (■), and 4 days (▲) after infection. Each point is the mean of 5 measurements ± standard error.

was determined at various time intervals following a single intraperitoneal dose of radioiron. Uninfected and infected mice were injected with radioiron at the same time, the infected mice being given 10 LD_{50} of MHV-3 virus 24 h earlier. Counts were taken at 2, 6, 12, 24, 48 and 72 h after radioiron was administered.

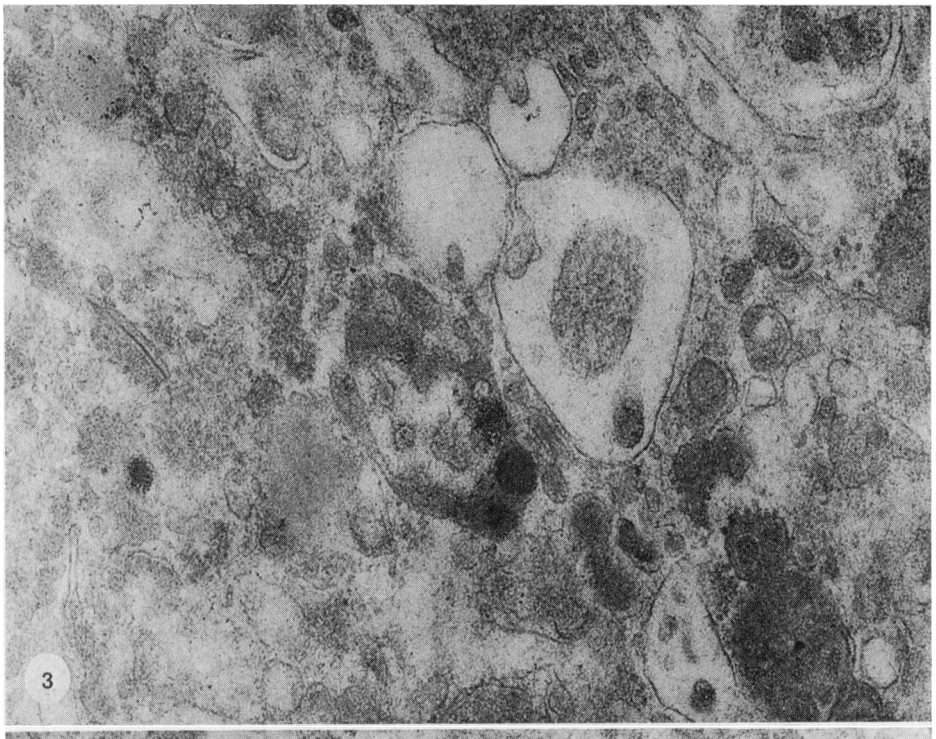
As shown in Fig. 1, the uninfected mice showed an initial increase in the level of iron uptake until it reached a maximum in 6 h. Thereafter the counts decreased and reached a low point in 72 h. By contrast, the infected mice, although following essentially the same pattern as uninfected mice up to 24 h, began to show an increase in iron uptake by 24 h and continued to increase steadily until 72 h when the experiment was terminated.

In the next series of experiments an attempt was made to compare the course of iron uptake when a single dose of iron was administered to mice at various stages of infection. For this purpose a mixture of 2 μCi of ^{59}Fe and 140μg $FeSO_4$ was administered to groups of mice which were previously infected with 10 LD_{50} of MHV-3 virus for 1, 2, 3 and 4 days. Radioactive counts were measured at 2, 6, 12 and 24 h after radioiron was administered.

As can be seen in Fig. 2, differences were detected in time intervals at which the administered iron peaked in the livers. In one- and two-day infections as well as in uninfected mice, the liver iron peaked in 6 h after administration. By contrast, in the 3- and 4-day infections, sharp peaks were noticed in the administered iron in 2 h. Generally, the iron levels tended to decline after the initial peak. The erratic second iron peak at 24 h for the three-day infection is not readily explainable.

Pathological alteration of infected livers

Examination of liver lesion was performed by haematoxylin-eosin staining. Little or no pathological changes were observed on tissue section obtained one day post infection. However, after the second day of infection some inflammatory reactions were noted. In general, these reactions were characterized by infiltration of polymorphonuclear cells, increased mitosis, and nuclear degeneration of the hepatocytes, *viz.*, nuclear pyknosis, karyolysis and karyorrhexis. Well-defined focal narcotic lesions were observable on the third day of infection. The necrotic foci increased in number on the fourth day of infection resulting in extensive damage to hepatocytes.



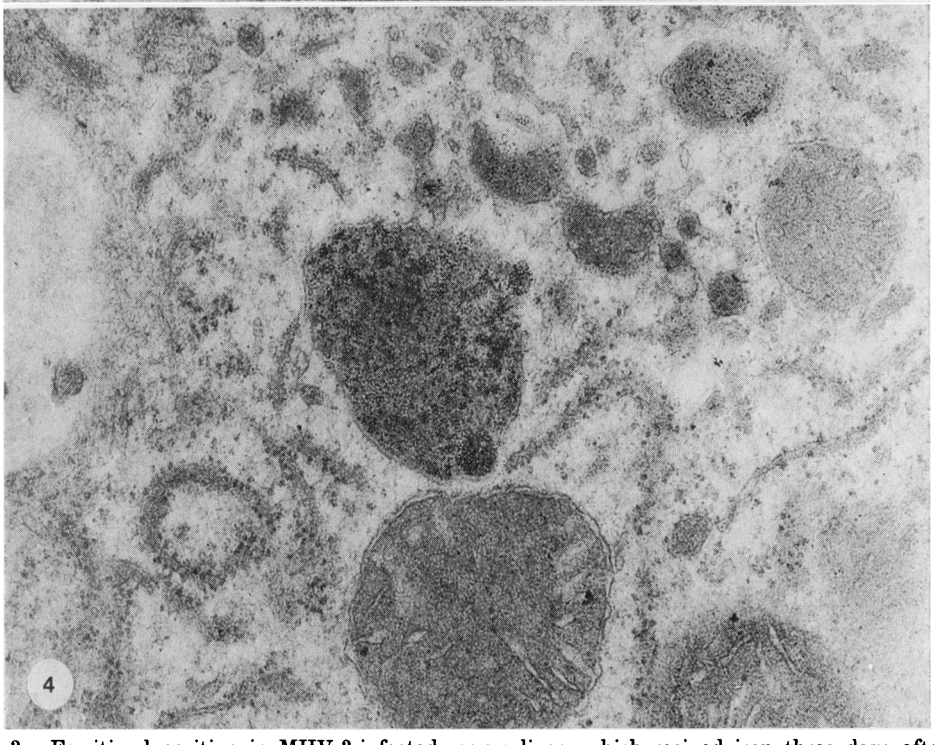


FIG. 3.—Ferritin deposition in MHV-3-infected mouse livers which recived iron three days after infection. Aggregated electron-dense micelles are observed in the dilated sac of endoplasmic reticulum. Fixation: glutaraldehyde and OsO_4. (Uranyl acetate and lead citrate. × 43,125.)

FIG. 4.—Ferritin deposition in MHV-3-infected mouse livers which received iron 4 days after infection. A predominant deposition of aggregated electron-dense micelles of ferritin is noted in the degenerative mitochondria. Fixation: glutaraldehyde and OsO_4. (Uranyl acetate and lead citrate. × 37,500.)

Immunofluorescence of viral antigen

MHV-3 antigen in infected livers was demonstrated by indirect immunofluorescent antibody technique, using mouse anti-MHV-3 and fluorescein-conjugated rabbit anti-mouse IgG. Slight fluorescence was observed on the first day of infection which progressed to 3+ intensity on the second day and third day of infection, and began to diminish on the fourth day of infection.

Electron microscopy for ferritin micelles

Accumulation of ferritin micelles was noted 24 h after iron administration on the third day of MHV-3 infection, as shown in Fig. 3. In contrast, no ferritin micelles could be observed in uninfected cells.

An electron micrograph of cells of iron-injected mouse (Fig. 4), which was previously infected for 4 days, showed ferritin-like electron-dense micelles in the degenerated organelles which appeared to be mitochondria due to the presence of a double membrane.

DISCUSSION

The results described in this work demonstrate a higher degree of iron uptake in MHV-3 infected mouse livers than in uninfected livers. In the uninfected mice, the peak of iron uptake occurred at 6 h after injection of iron, which is in general agreement with a similar experiment in rats (Gardiner and Morgan, 1974). In our experiments, when mice were injected with iron on the 3rd or 4th day of infection, peak of iron uptake occurred 2 h after injection of iron. The concomitant increase in iron uptake in the 3- and 4-day infected livers correlates well with the degree of necrosis of liver sections examined by haematoxylin-eosin staining. In the haematoxylin-eosin staining sections, we found a number of polymorphonuclear cells infiltrated to the necrotic lesions. Such being the case, it is reasonable to suspect that the infiltrated polymorphonuclear cells may have participated in active iron uptake. As shown in Fig. 2, peak uptake began to shift from 6 to 2 h after iron administration on the 3rd day of infection, and was markedly significant on the 4th day of infection ($P=0{\cdot}053$, one-tailed test).

An attempt was made to compare by calculation the rate of uptake of carrier iron in the livers of mice at various stages of infection. The amount of carrier iron was computed on the basis of the radioactive ct/min of ^{59}Fe in the livers which were compared with ct/min of the iron dose injected into the mice. In the uninfected mice, the amount of carrier iron uptake was 47·23 μg at 2 h and 57·35 μg of $FeSO_4$ at 6 h or 33·74% and 40·96% respectively of the total injected iron. By comparison, the amount of carrier iron in mice with 3-day infection was 63·45 μg (45·30%) at 2 h and 6 h, and similarly 80·96 μg (57·83%) at 2 h and 70·17 μg (50·12%) at 6 h in the 4-day infected livers. Thus, the average increases in the 3- and 4-day infected livers were 1·34 and 1·62 times greater than uninfected livers.

Electron microscopic study of liver sections revealed electron-dense ferritin micelles aggregated in the dilated sac of the endoplasmic reticulum. Electron micrographs of 4-day infected livers showed ferritin micelles in free and aggregate forms. Interestingly, both forms of ferritin were found in the mitochondria of 4-day infected livers. The presence of mitochondrial ferritin is very rare in normal humans but it is present constantly in certain human pathological states such as refractory hypochromic anaemia, thalassaemia and other anaemias (Bessis and Brenton-Gorius, 1972; Cartwright and Deiss, 1975). Our observed increase of ferritin synthesis in MHV-3-infected livers may involve acute-phase reacting proteins and iron retention caused from an inflammation (Konijn and Hershko, 1977).

The results reported here show a fair degree of correlation between iron retention and the extent of hepatic injury.

This is in keeping with the observations in chemically induced inflammation in rat livers and spleens (Konijn and Hershko, 1977) and in patients with liver disease (Prieto *et al.*, 1975). The increase in iron deposition may have been due to the alteration in iron metabolism in the progressively MHV-3 damaged cells.

It was speculated that increased serum iron levels, as seen in patients with chronic anicteric hepatitis of Down's syndrome, were a function of the presence of Australia antigen rather than a characteristic of liver damage (Sutnick *et al.*, 1974). However, in the absence of sufficient data in Sutnick's work, the probability of this event seems to us to be remote. The results obtained by our experiments point to a secondary effect of liver cell injury rather than a direct result of viral infection.

It is of more than passing interest to note that *in vivo* splenic iron uptake by mice infected with Friend polycythaemia virus was shown to be greatly increased due to erythropoiesis (Hankins *et al.*, 1973; Mirand *et al.*, 1961). However, Hankins and co-workers were unable to account for the excess iron uptake on the basis of haemoglobin-supplied iron alone. On the basis of indications from our results, it can be argued that the observations of Mirand *et al.* and Hankins *et al.* may have been due to splenic injury caused by viral infection in addition to erythropoietic effect.

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