encephalitis virus, another group B arbovirus. In their studies, it was shown that the virus could persist in cell cultures for 21 days. However, the infected cells were not serially propagated and therefore could not be considered as chronically infected.

In this report, it was shown that no special procedures were necessary to maintain the balance between L-929 cellular multiplication and WNV replication. Morphological changes in the infected cells were seen in subcultures and infectious virus was consistently present in the culture medium.

Summary. When West Nile virus was added to cultures of L-929 mouse fibroblasts, characteristic morphological changes were produced. The infected cultures were not destroyed although infectious virus was consistently demonstrated in the growth medium. Infected cells were readily subcultured and the virus–cell relationship was continued without any alteration in cultural procedures.

1. Ginsberg, H. S., Progr. Med. Virol. 1, 36 (1958).
2. Morgan, H. R., Probl. Virol. (USSR) (English Transl.) 4, 1 (1959).
3. Walker, D. L., Progr. Med. Virol. 6, 111 (1964).
4. Earle, W. R., J. Natl. Cancer Inst. 4, 165 (1943).
5. Smithburn, K. C., Hughes, T. P., Burke, A. W., and Paul, J. H., Am. J. Trop. Med. 20, 471 (1940).
6. Eagle, H., Science 130, 432 (1959).
7. Reed, L. J. and Muench, H., Am. J. Hyg. 27, 493 (1938).
8. Chambers, V. C., Virology 3, 62 (1957).
9. Hearn, H. J. and Brown, A., J. Bacteriol. 78, 595 (1959).
10. Dubbs, D. R. and Scherer, W. F., J. Bacteriol. 91, 2349 (1966).

Received June 25, 1968. P.S.E.B.M., 1968, Vol. 129.

Increased Susceptibility to Murine Hepatitis Virus Infection by Treatment with Iron Salts* (33386)

JOEL WARREN, RACHEL J. MASON, LUIZ H. BARBOSA, KAREN L. GABBARD, AND MARVIN BUCY

Chas. Pfizer & Co., Inc., Department of Biologics Research, Terre Haute, Indiana 47808

This study was undertaken to determine whether susceptibility of mice to a viral hepatitis infection could be modified by the administration of iron salts. Ferric ammonium citrate (FAC) was selected since large doses are capable of inducing centrilobular hepatic necrosis (1), and because Bullen, *et al.* (2) cite their own observations as well as those of others (3) in support of the thesis that active and passive immunity to several bacterial infections in rodents can be abolished by the inoculation of iron salts. These reports suggest that this may be related to saturation of transferrin, a bacteriostatic iron-binding serum protein (4), and not to a direct effect of iron on the microorganisms. The present paper describes an enhanced susceptibility in mice to mouse hepatitis virus (MHV) following the inoculation of soluble iron and the protective effects of a chelating agent, EDTA, against this disease.

Materials and Methods. Virus. MHV-3 was obtained from Dr. M. Pollard in an infected primary explant of mouse embryo. It was passed intraperitoneally in day-old mice and their livers were harvested 5–7 days later when macroscopic lesions were prominent. A clarified 10% homogenate of this tissue in Eagle's basal medium + 10% fetal calf serum was frozen at —60° and served as a standard inoculum.

Mice. Swiss albino mice, originally of a hybrid NIH stock, were obtained from a local dealer. Because maturing animals are increasingly resistant to high doses of MHV, newborn litters 1–2 days of age were used exclusively. Randomized sucklings from

* This investigation conducted under U.S. Army Contract No. DA-49-193-MD-2364.

TABLE I. Relation of Interval after Treatment to Effect of FAC on Resistance to MHV.

FAC (mg/kg, i.p.)	Interval before infection	MHV (ID_{50}/ml, i.p.)	Mortality		Log ID_{50}
			No.	(%)	
0	—	300	22/22	100	
	—	30	12/22	54	3.2
	—	3	4/21	11	
5	20 min	300	16/18	88	
		30	15/18	83	3.5
		3	2/20	10	
5	24 hr	300	21/21	100	
		30	19/19	100	>4.0
		3	20/21	95	
5	—	—	2/23	9	—
30	—	—	1/21	4	

several litters were combined with several foster mothers to provide 15–20 mice/test group.

Tissue cultures. Monolayer cultures were prepared from trypsinized whole mouse embryo or from mouse macrophages obtained by peritoneal gavage 48 hr after an intraperitoneal injection of sterile 1% cornstarch. All cultures were grown in Leighton tubes and fed with medium 199 + 20% fetal calf serum.

Iron salt. Ferric ammonium citrate (Mallinckrodt), 17.5% iron, and ethylenediaminetetracetic acid (EDTA)-disodium salt (Eastman) were dissolved in distilled and deionized water.

Results. Effect of iron administration on MHV infection. Preliminary toxicity studies performed in mice of different ages indicated that a single i.p. dose of between 5.0 and 30 mg/kg of FAC was well tolerated with an overall mortality of less than 8% in 1–2-day-old mice. A series of 9 experiments was then performed in which animals of different ages were treated with a single dose of FAC 20 min or 24 hr prior to the i.p. inoculation of varying amounts of MHV. Table I illustrates results of a typical experiment. The FAC only slightly increased the LD_{50} when administered 20 min before infection, whereas the infectivity of MHV was significantly increased when an interval of 1 day elapsed before virus challenge. Injection of FAC also shortened the incubation period of illness and increased the lethality of a given dose of virus (Fig. 1).

Gross and microscopic examination of the livers of treated animals revealed a marked increase in the extent and severity of hepatitis and particularly the development of small focal lesions on the liver surface. The FAC alone in the doses employed produced no pathological changes of the liver detectable by hematoxylin and eosin staining and the Prussian blue stain for ferric iron was consistently negative in liver sections of treated and control animals. The virus content of the liver of iron-treated animals was markedly increased and detectable levels were observed earlier in the disease when compared with those in the controls (Table II).

Our strain of MHV-3 did not produce clinical illness in weanling mice even though virus could be isolated from the livers of newborn infected animals when sacrificed at 10–14 days of age. It was therefore of interest to find that injection of ferric iron did not modify the maturation barrier (Table III). In other experiments mice were given two doses of FAC (30 mg/kg each) at 2-day intervals and then challenged 24 hr after the last dose. Again the adult animals completely resisted the virus challenge. Because maximum enhancement of susceptibility occurred when there was a delay between the injection of iron and virus infection, we investigated the effect of an insoluble salt, ferric oxide (Fe_2O_3), on MHV infection. The i.p.

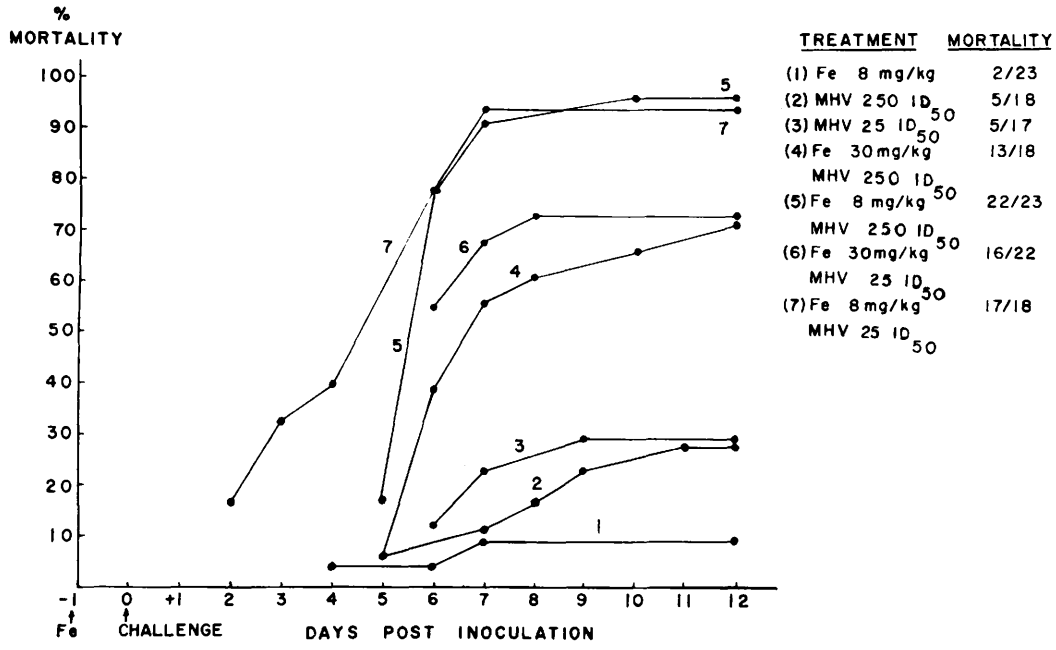

FIG. 1. Effect of ferric ammonium citrate on mouse hepatitis infection; soluble iron.

inoculation of this material 1 or 2 days prior to virus challenge did not modify the course of illness (Fig. 2).

Effect of FAC on MHV in tissue culture. The search for a biochemical basis for the effect of iron might be simplified were it possible to demonstrate a similar potentiating action in cell cultures *in vitro*. Therefore, MHV was titrated in cultures of whole mouse embryo prepared from tissues obtained 24 hr prior to birth. To these was added FAC at the nontoxic level of 0.05 mg/ml. The presence of FAC did not modify either the titration end point ($10^{-4.5}$ $TCID_{50}$/ml) or the development of CPE which occurred in approximately 3 days. In other experiments mouse macrophages from normal or FAC-treated adult donors and normal macrophage cultures to which FAC was added were compared for their sensitivity to MHV. Again there was no effect of the iron and the virus multiplied to a similar titer ($10^{-3.2}$) in all cultures.

Protection of mice against MHV by a chelating agent. Sword observed that the enhancing effect of Fe^{2+} on *L. monocytogenes* infection could be reduced by the prior administration of a chelating agent (5). To determine whether the effects of iron salts were related to serum iron levels, we investigated

TABLE II. MHV Concentration in Liver Homogenates from Mice Treated with FAC and Controls.[a]

Infecting dose (ID_{50}/ml)	FAC (mg/kg)	Log ID_{50}/g of liver on day									
		3	4	5	6	7	8	9	10	11	12
350	—	<0.5	<0.5	1.0	0.5	1.8	—	2.5	2.5	<2.0	—
	30	<0.5	1.6	2.2	2.4	2.9	—	3.6	4.0	3.0	—
35	—	<0.5	<0.5	0.5	1.6	1.8	1.5	2.0	<1.0	<1.0	<1.0
	30	<0.5	<0.5	2.4	3.6	3.7	4.0	4.0	4.2	2.0	<1.0

[a] Five mice from each group were sacrificed on day shown and pooled livers were homogenized to a 10% suspension which was frozen until titrated in day-old mice.

TABLE III. Lack of Effect of Ferric Ammonium Citrate on Age Susceptibility to MH Virus.[a]

FAC (mg/kg)	Age (days)	MHV (ID_{50})	Survival (days)								Mortality	
			1	2	3	4	5	6	7	8	No.	(%)
0	1	500			2	7	4	4	3	2	22/22	100
	1	50		1		3	1	5	3	4	16/18	88
	1	5						1	2	1	4/21	11
5	1	500			6	11	1	1	1		20/20	100
	1	50		2	9	5	1	1			18/18	100
	1	5			4	4	2	4	1		15/20	75
0	7	500				1	4	1	6		12/20	60
	7	50			1			3			4/19	21
5	7	500		2	3	1	3	3	3		15/18	83
	7	50		1	1	7	1	3	1	1	15/18	83
0	14	500					1	1			2/20	10
	14	50									0/19	0
5	14	500				1	2		1		4/21	19
	14	50									0/20	0

[a] FAC and virus administered i.p. 24 hr apart in 0.1 ml.

EDTA-disodium salt for its ability to modify the effects of FAC. This chelating agent was of low toxicity and single i.p. doses of 250–500 mg/kg were completely tolerated by day-old mice. To ensure that EDTA itself was not virucidal 1 mg of EDTA was incubated *in vitro* with 300 ID_{50} of MHV for 1 hr at 37° and the mixtures were then tested in newborn mice. There was no loss of virus infectivity. In the first experiment groups of newborns received 30 mg/kg of FAC and on the following day 50 or 500 mg/kg of EDTA was injected i.p. and followed by live MHV 6 hr later. The EDTA failed to counteract the

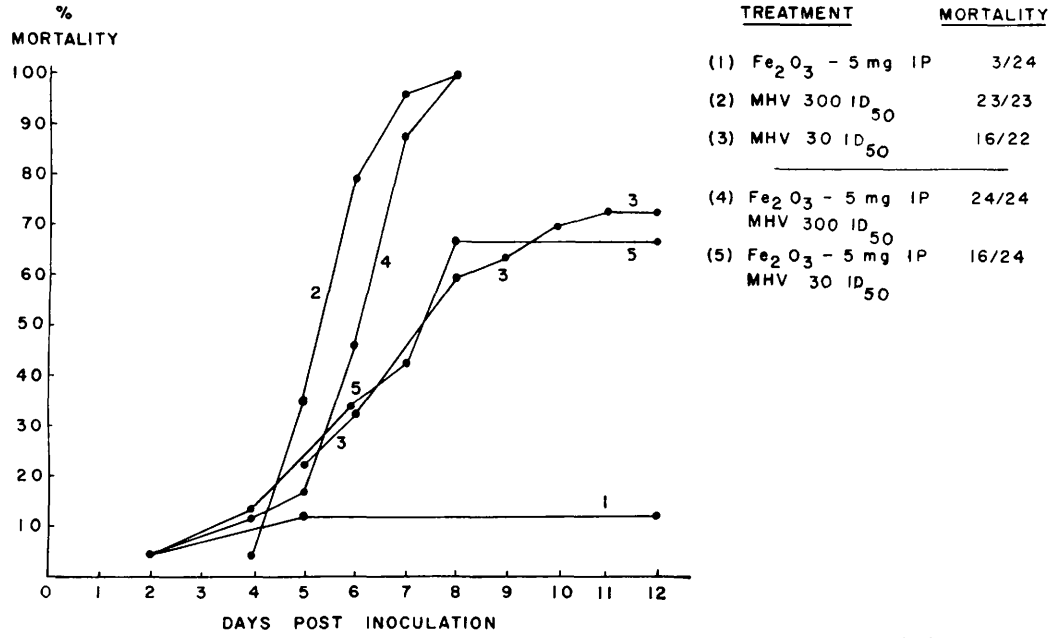

FIG. 2. Effect of ferric oxide (Fe_3O_8) on mouse hepatitis infection; insoluble iron.

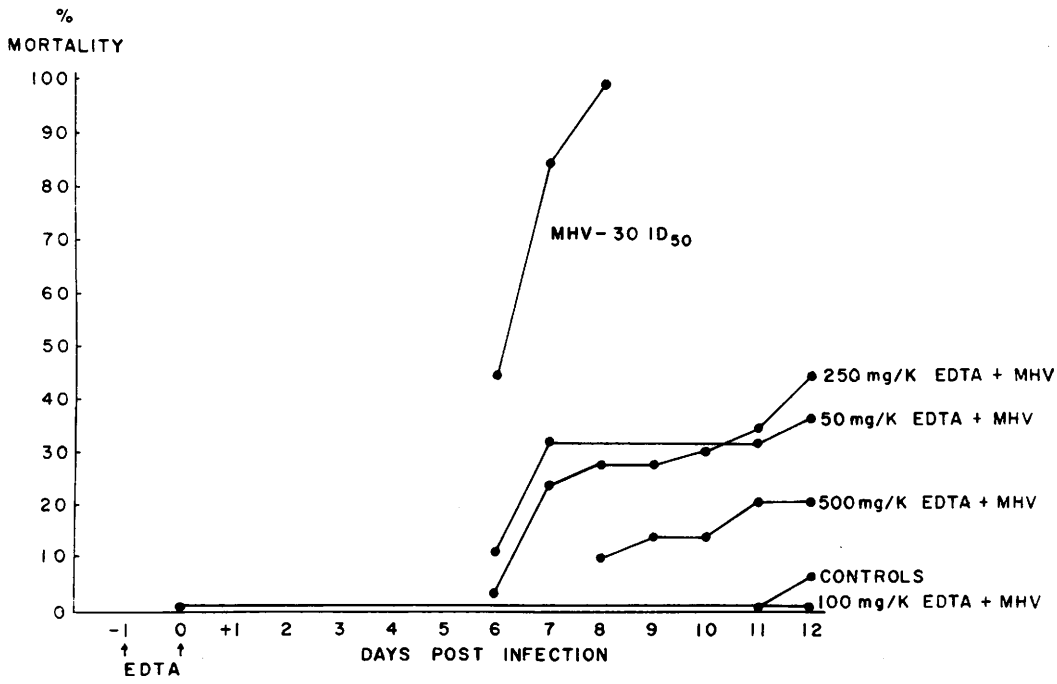

FIG. 3. Effect of EDTA on MHV infection.

effect of ferric iron and the mortality of the FAC-treated group exceeded that of the controls by the usual margin. However, it was noted that the MHV mortality in the mice that received EDTA alone was significantly less than that in the untreated controls. This observation led to a series of experiments to determine whether the administration of EDTA to normal newborn mice would enhance their resistance to MHV. Groups of 20–30 mice 1 day old were inoculated i.p. with varying amounts of EDTA 6 hr prior to infection with a dose of MHV (10^{-3}) calculated to kill 80–100% of the control animals. Typical results from a set of 4 experiments (shown in Fig. 3) demonstrate considerable protective effect against this viral hepatitis. The age of the mouse was critical since EDTA administered to suckling animals at 3–5 days of age conferred no significant prophylactic effect.

Attempts to infect monkeys with MHV after administration of iron. On the possibility that the increased susceptibility to MHV in iron-treated mice might also occur in primates given FAC, we inoculated groups of two monkeys each with FAC at 30 and 60 mg/kg administered four times 48 hr apart. Two days after the last dose these animals and two untreated controls were given 2 ml i.p. of a 10% suspension of mouse liver having an MHV titer of $10^{-4.5}$. The daily temperatures remained within a normal range for 30 days, at which time the monkeys were sacrificed. No evidence of viral infection was found in sections of lung, heart, liver, spleen, and kidney.

Discussion. Murine hepatitis was selected for these experiments because it is primarily a disease of the liver parenchyma with minimal involvement of other organ systems (6). The studies of Gallily *et al.* (7) indicated a genetic basis for susceptibility. However, this could be altered by the administration of cortisone which decreased resistance to MHV (8). Since cortisone elevates serum iron (9), this hypersideremia could be responsible for enhancement of the virulence of MHV.

The action of FAC on a viral disease of newborn mice involves a delay of several hours before it is effective. This suggests that uptake of ferric iron, reduction to the ferrous state, and saturation of transferrin may be involved in the potentiation of virus infec-

tivity. Failure of FAC to increase the viral susceptibility of murine embryonic and macrophage cells in culture also points to a humoral factor in this phenomenon.

The ability of EDTA, a chelating agent, to protect newborn mice against MHV was of considerable interest. Although we have assumed that this was due to a reduction of serum iron levels and essentially the converse of the effect of parenteral FAC, no biochemical studies were performed and one cannot rule out a possible decrease in ionized calcium or other ions in extracellular fluids as a factor in its prophylactic action.

Infectious hepatitis in man is distinguished by profound derangements of iron metabolism. To quote Peterson (10), "Acute hepatitis is the only disease in human beings consistently associated with a marked hypersideremia. There is no satisfactory evidence to explain this." Further, there is a significant decrease in cytochrome oxidase in acute viral hepatitis (11). Whether a reduction of serum iron by the administration of a chelating agent will alter susceptibility or the course of IH in man merits investigation.

Summary. The administration of ferric ammonium citrate to newborn mice enhanced their susceptibility to MHV. Intraperitoneal inoculation of a chelating agent, EDTA, protected a significant proportion of newborn animals against MHV but only when administered shortly after birth.

1. Large, F. C., Am. J. Clin. Pathol. **35**, 427 (1961).
2. Bullen, J. J., Rogers, H. J., and Cushnie, G. H., Nature **214**, 515 (1967).
3. Martin, C. M., Jandl, J. H., and Finland, M., J. Infect. Diseases **112**, 158 (1963).
4. Schade, A. L. and Caroline, L., Science **104**, 341 (1946).
5. Sword, C. P., J. Bacteriol. **92**, 536 (1966).
6. Nelson, J. B., Proc. Soc. Exptl. Biol. Med. **120**, 41 (1965).
7. Gallily, R., Warwick, A., and Bang, F., J. Exptl. Med. **125**, 537 (1967).
8. Gallily, R., Warwick, A., and Bang, F., Proc. Natl. Acad. Sci. U. S. **51**, 1158 (1964).
9. DeMulder, R., A. M. A. Arch. Internal Med. **102**, 254 (1958).
10. Peterson, R. E., J. Lab. Clin. Med. **39**, 225 (1952).
11. Sherlock, P. E. and Walshe, W. A., J. Pathol. Bacteriol. **59**, 615 (1957).

Received June 25, 1968. P.S.E.B.M., 1968, Vol. 129.

Endotoxin-Induced Inhibition of Renal Function in the Mouse (33387)

H. M. BATES AND S. MARGOLIN
Wallace Laboratories, Division of Carter-Wallace, Inc., Cranbury, New Jersey 08512

The literature dealing with the effects of endotoxin on kidney function is surprisingly limited. In a number of recent reviews on the action of endotoxin on the host, the involvement of the kidney in endotoxemia has not been taken into consideration (1–3). Renal dysfunctions, however, have been reported to occur in dogs and monkeys following an injection of endotoxin (4, 5), and in humans after the administration of blood contaminated with gram-negative bacilli (6). In mice, Berry and Smythe (7) found that endotoxin inhibits the ACTH-induced increase in urinary nitrogen excretion, and, in a later study (8), they showed that these findings were related to impaired renal function.

The present study not only confirms and extends the findings of Berry and Smythe that renal function is depressed in mice treated with appropriate doses of endotoxin, but also demonstrates that mice can be rendered tolerant to the renal inhibitory effects of endotoxin. In addition, experiments are described which show that the renal effects of endotoxin can be quantitatively determined by measuring the blood, brain, and liver concentrations of urea nitrogen, the quantity of urea nitrogen excreted, and the renal clear-