ONTOGENY OF MACROPHAGE RESISTANCE TO MOUSE HEPATITIS IN VIVO AND IN VITRO*

By RUTH GALLILY,‡ ANNE WARWICK,§ and FREDERIK B. BANG, M.D.

(From the Department of Pathobiology, The Johns Hopkins University School of Hygiene and Public Health, Baltimore, Maryland)

Plates 70 and 71

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The genetic resistance of mice to the mouse hepatitis virus resides in macrophage cells, since cells identified as macrophages from resistant animals are resistant in vitro, and in contrast macrophages from susceptible mice are rapidly destroyed in vitro. In our first report on this subject (1) a comparison was made between the macrophages derived from liver cultures of infant C_3H mice, which as weanling mice are resistant to the virus, with liver macrophages derived from Princeton Swiss (PRI) mice, which as weanlings remain highly susceptible. A close correlation between the susceptibility of the macrophages and the mice was found in F_1 and F_2 hybrid generations and various backcross generations (1). Subsequently, extensive tests with macrophages obtained by peritoneal washings from young adult mice confirmed the relationship between in vitro and in vivo susceptibility (2). The previous studies ignored the question of the susceptibility of infant C_3H mice (resistant strain) themselves. Therefore, it was thought worthwhile to study this point, and if found, the change of the susceptibility with the age of the animal. Likewise, in parallel with the in vivo experiments it seemed advantageous to check in vitro the gradual manifestation of this genetic resistance with age.

Materials and Methods

Virus.—The mouse hepatitis virus (MHV) had been originally obtained from Dr. John Nelson of The Rockefeller University and was maintained in a PRI inbred strain of mice. The virus was harvested from the livers of infected 3- to 4-wk-old PRI mice. A 10% suspension of infected liver was prepared in Hanks' balanced salt solution (BSS). Ampoules of the virus were kept at −30°C. The titer (LD_{50}) of the virus was determined in 3- to 4-wk-old PRI mice. The virus will be referred to as MHV(PRI). Virus partially adapted to C_3H mice and harvested from the liver of infant C_3H mice will be referred to as MHV(C_3H).

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‡ Present address: Section of Cell Biology, The Weizmann Institute of Science, Rehovoth, Israel.

§ Deceased 11 November 1965.

Mice.—The C_3H strain was originally obtained from Dr. H. B. Andervont of the National Cancer Institute as a strain free of the milk agent. It has been inbred in this laboratory for 69 generations. The PRI strain of mice was obtained from Dr. John Nelson in 1954 and has maintained, as an inbred strain, for 28 generations.

TABLE I

The Percentage of Mortality of Infant C_3H Mice after MHV (PRI) Intraperitoneal Injection

Age	Dilution of MHV (PRI)						
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
days							
1–2	100	100	83	75	52	0	0
3–4	100	100	83	75	66	0	
5–6	100	86	50	83	0		
7–8	61	44	33	27	13	0	
9–10	90	54	40	0			
11–12	46	20	27	9	0		
13	23	0					
14	0						
15	0						

TABLE II

Survival Time of Infant C_3H Mice after Intraperitoneal Injection of MHV (PRI)

Age of mice	Average time of death after inoculation, *days**				
	Dilution of MHV				
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
days					
1–2	6.5 ± 0.8	6.7 ± 0.6	7.6 ± 0.5	7.7 ± 0.5	6.4 ± 0.5
5–6	7.5 ± 0.5		7.5 ± 0	7.0 ± 0.3	
7–8	7.7 ± 0.4	7.0 ± 0	8.0 ± 0	7.0 ± 0	8.0 ± 0.2
11–12	7.0 ± 0.5	7.0 ± 0	8.0 ± 0		

* The experiments were carried out with groups of 6–18 animals each.

Macrophages from Liver.—Small pieces of liver, about 1 mm square, from 1- to 8-day-old mice, were placed on a layer of reconstituted rat tail collagen (3, 4), in roller tubes, in the roller drum. Chang's medium was used to culture the liver macrophages. This medium (5) contains 90% inactivated horse serum, 2% beef embryo extract, and 8% Hanks' BSS. The tubes were kept for 25–30 days. The media were changed, in most of the experiments, 7 days after virus was added, and every 4–6 days thereafter. In a study to be reported elsewhere, Huang (J. H. Huang, unpublished data) has found that certain horse sera contain inhibitors for MHV. The results reported are limited to sera from individual horses known to be free of inhibitor.

RESULTS

Responses of infant C_3H mice to MHV (PRI).—Infant C_3H mice, 1–15 days old, were injected intraperitoneally with varying concentrations of MHV (PRI) (10^{-2}–10^{-8} dilutions of stock virus) and kept under observation for 1 month. The original titer of MHV (PRI) in PRI animals was $10^{8.3}$ (LD_{50}). The results

TABLE III

Susceptibility of Macrophages from C_3H Livers to MHV(PRI)*

No. of experiment	Age of mouse from which liver was taken	Time of destruction (No. of days after adding MHV)	Macrophage description‡		MHV recovered from supernatant	
			No. of tubes with destruction	Degree of destruction	Destruction	
					Present	Absent
	days			%		
2	1	12	2/3	75	§	
9	1	12	3/3	75	+	
12	1	15	2/3	50–100	+	
15	1	14	2/4	50–75	+	
21	1	11	5/6	75–100	+	
10	3	21	0/4			−
13	3	14	3/4	50–75	+	
14	3	17	1/4	50	+	
17	3	18	0/4			−
19	5		0/6			−
20	5	15	1/8	50	+	
24	5		0/8			−
25	5	21	1/5	50	+	
22	8		0/8			−
23	8		0/8			−

* The livers were taken from C_3H mice of different ages.

‡ The cultures were kept for 30 days.

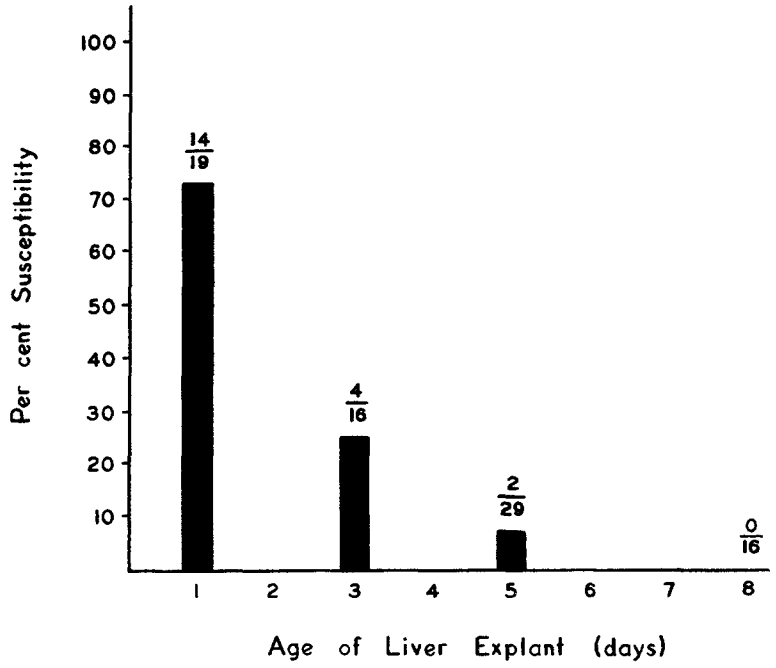
§ Not tested.

are summarized in Table I. The data in the table indicate that newborn C_3H mice are highly susceptible to the virus and that the resistance of the C_3H mouse to MHV increases with its age. 14-day-old mice were, as previously indicated for weanlings (2), fully resistant to a standard inoculation of the virus (0.1 ml of a 10^{-2} dilution of the stock virus suspension).

The survival time of infant C_3H mice, infected with different dilutions of MHV(PRI), was 6.5–8.5 days and it did not vary significantly either with the age of the animals or with the dilution of the virus (Table II). As this survival

time exceeds that of the susceptible PRI mice (2–4 days) these infant C_3H mice may be called delayed susceptible.

Susceptibility of C_3H Liver Macrophages to MHV(PRI) in Vitro.—After observing that infant C_3H mice had a delayed susceptibility to MHV(PRI), it seemed important to find out if liver macrophages of newborn C_3H mice in vitro would also show delayed susceptibility to the virus.



TEXT-FIG. 1. Susceptibility of C_3H liver macrophages from mice of different ages.

In a series of different experiments, livers were taken from 1-, 3-, 5-, and 8-day-old C_3H mice. In culture maintained in 90% serum, parenchymal liver cells grew poorly, but macrophages seemed to thrive (Fig. 1). Virus was added (0.1 ml/tube of 10^{-2} virus dilution) to the culture tubes at 3 days of culture, at which time moderate numbers of macrophages had migrated from the liver pieces on collagen to the glass surface of the tubes. Changes in the cells were noted during 30 days of observation, and the supernatant was tested for MHV by injecting it into infant C_3H or adult PRI mice.

The results are presented in Table III and Text-fig. 1. The data show that the susceptibility of C_3H mouse liver macrophages to MHV(PRI) decreased with the increase in the age of the donor animal. Susceptibility was indicated when about 75–100% of cells were destroyed. While 73% of the cultures derived

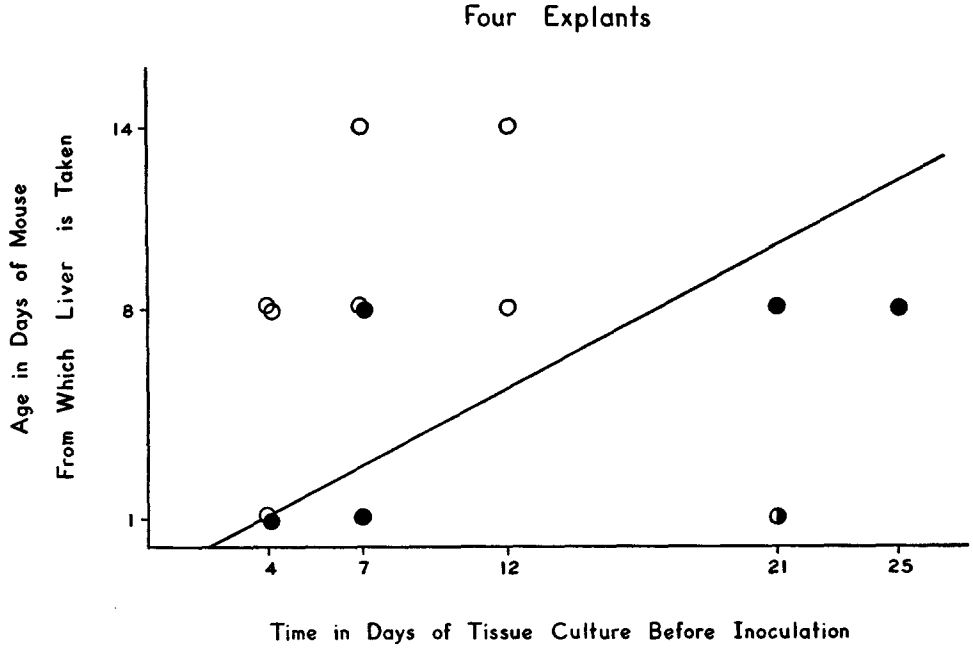
from 1-day-old liver were susceptible, only 7% of the cultures derived from 5-day-old liver and none of those from the 8-day-old liver were susceptible to this virus. (Compare Figs. 1 and 2 with Figs. 3 and 4.)

The Effect of Age and Number of C_3H Liver Macrophages In Vitro and Their

TABLE IV

Susceptibility of C_3H Liver Macrophages from 1-Day-Old Mice to MHV (PRI), after Different Times of Growth In Vitro

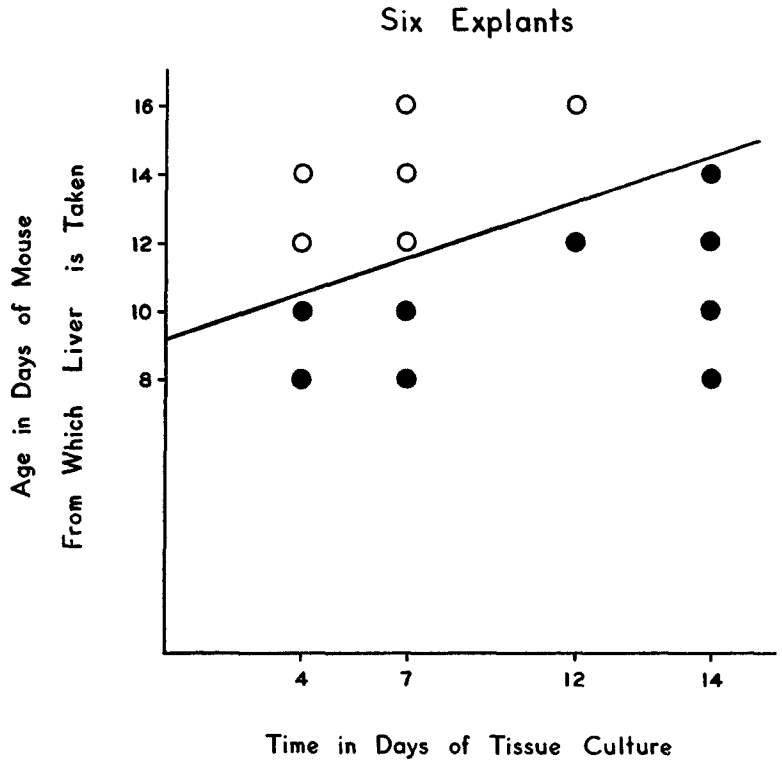
Culture	Time after which virus was added to culture. *days*					
	3		7		10	
	Destruction		Destruction		Destruction	
	No. of tubes	%	No. of tubes	%	No. of tubes	%
1-day-old C_3H liver	5/10	50	7/7	100	10/10	100



TEXT-FIG. 2. ○, none of the inoculated cultures showed destruction; ●, all of the inoculated cultures showed destruction; and ◑, a fraction of the cultures showed destruction.

In most cases each point represents three cultures; in a few only two cultures were inoculated.

Susceptibility to MHV(PRI).—Macrophages cultured in these first experiments from older mice (8 days old, see Table III) were resistant. This maturation of resistance apparently took place in the animal. Experiments therefore were undertaken to determine if susceptible liver macrophages become resistant after being cultured in vitro for different lengths of time; i.e., will maturation or ontogeny of resistance proceed in vitro? Pieces of C$_3$H liver from 1-day-old

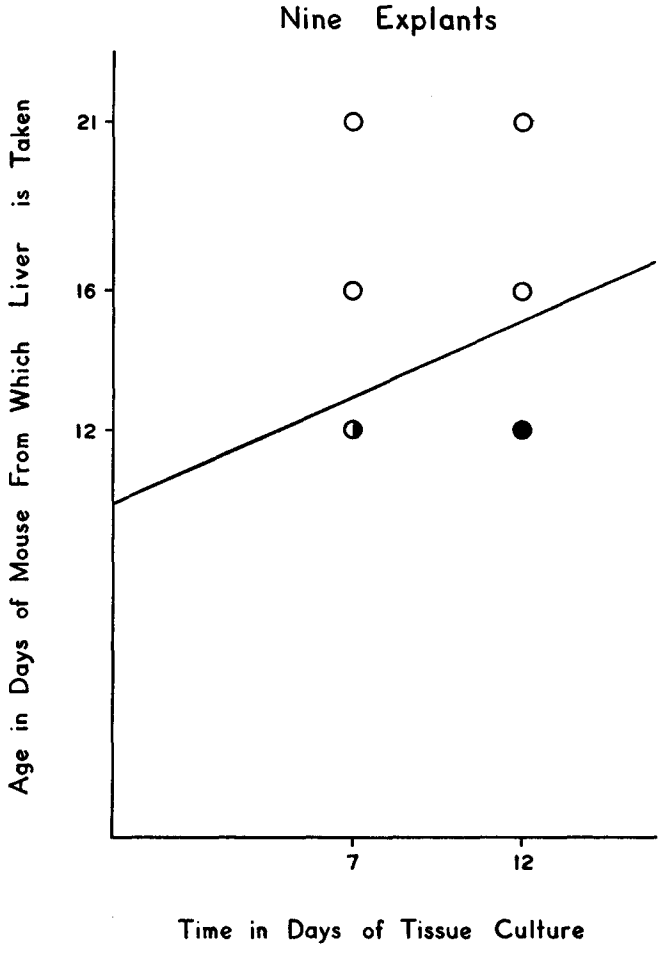


TEXT-FIG. 3. Symbols as in Text-fig. 2.

mice were placed on collagen as before. MVH(PRI) was added to the tubes after 3, 7, and 10 days had elapsed. The results are summarized in Table IV. Continued growth in vitro did not increase the resistance of the liver macrophages to MHV(PRI) and indeed may have decreased resistance.

In a previous study of the effect of cortisone on the resistance of C$_3$H macrophages to MHV(PRI) (6) it had also appeared that cultures maintained in vitro for longer periods of time before testing for viral susceptibility became more susceptible. Since the number of macrophages seen in a culture seemed to in-

crease with time, the question was raised whether or not the number of cells in culture at the time of virus addition influenced the susceptibility. To test this a series of experiments with different numbers of explants, which presumably



TEXT-FIG. 4. Symbols as in Text-fig. 2.

produce different numbers of macrophages per culture tube, were compared for susceptibility. As may be seen by comparing Text-figs. 2, 3, and 4, increased length of time in culture and increased number of explants in culture reduced the resistance of the macrophages to MHV(PRI). However, all three figures show that with increasing age of mice from which the cells are derived, there is

increased resistance. It would appear that the resistance of cells to MHV in tissue culture, which reflects the ontogeny of resistance in mice, may be influenced by the number of cells in culture to some degree. Since these were cultures of liver, it is not known whether the apparent increase in number of macrophages with time or the increase in number of parenchymal liver cells or the total number of cells was responsible for this effect.

TABLE V

Susceptibility of C_3H Mice to MHV (PRI) after Successive Transfer of Virus in C_3H Infant Mice

Origin of MHV	No. of transfer in infant C_3H	Mortality		
		Infant* C_3H	Adult‡ C_3H	Adult PRI
PRI	0	6/6	0	3/3
Infant* C_3H	1	5/5	11/20	3/3
"	2	12/12	5/5	2/2
"	3	5/5	8/9	3/3
"	4	16/16	6/9	
"	6	9/9	7/11	
"	7	6/6	6/8	

* Infant C_3H mice were 3–5 days old.

‡ Adult C_3H mice were 30–45 days old.

TABLE VI

Susceptibility of Adult C_3H Mice and Adult PRI Mice to MHV Prepared from Adult C_3H Liver

Origin of MHV	Mortality	
	Adult C_3H	Adult PRI
Adult C_3H in 4th transfer	0/7	3/3
Adult C_3H in 5th transfer	0/12	3/3

One experiment with macrophages obtained from peritoneal washing of 12- and 16-day-old C_3H mice was performed. Partial destruction was produced with 10^{-2} and 10^{-3} dilution of virus.

Adaptation of MHV(PRI) to Adult C_3H Mice following transfer of MHV(PRI) to Infant C_3H Mice.—The delay in onset of both disease in infant mice and the time of killing of the cultures suggested that some adaptation of the virus was taking place in the C_3H animals and cultures. In three sets of experiments it appeared that following one transfer of MHV(PRI) to infant C_3H mice (4–5 days old) the virus became adapted to adult C_3H mice. About half of the adult C_3H mice were killed when injected with a 1:100 dilution of

virus prepared from 10% liver suspension of an infant C_3H mouse. In contrast, undiluted and diluted MHV(PRI) failed to kill C_3H adult mice. This preparation of MHV will be referred to as MHV(C_3H). Table V summarizes the mortality of infant C_3H mice, adult C_3H mice, and adult PRI mice after injection of different passages of MHV(C_3H) in C_3H infant mice. However, at no time did virus prepared from the infected livers of adult C_3H mice have the ability to kill other adult C_3H mice. The PRI mice remained susceptible to this adult C_3H preparation, and all mice so infected were killed (Table VI).

Table VII shows the survival time of infant C_3H mice, adult C_3H mice, and adult PRI mice after MHV(C_3H) injection. Infant C_3H mice died 2 days after MHV(C_3H) injection, while 7 days elapsed before MHV(PRI) killed C_3H mice of the same age (Table II).

TABLE VII

*The Survival Time of Mice after MHV (C_3H) Intraperitoneal Injection**

Infant C_3H (3–5 days)	Adult C_3H (30–45 days)	Adult PRI (30–50 days)
days	*days*	*days*
2.1 ± 0.3	5.2 ± 1	3.2 ± 0.7

* 0.01 ml of 10^{-2} dilution of liver suspension.

DISCUSSION

The genetic nature of the susceptibility of mice to one strain of mouse hepatitis has been established previously (2). Early work suggested that the factor was dominant and unifactorial (1). Since then a series of 12 backcrosses of susceptible C_3H hybrids to resistant C_3H mice have been done, and these continue to show that about 50% of the mice constantly furnish peritoneal washings which are susceptible to the virus (F. B. Bang, I. Vellisto, and A. Warwick, unpublished data). Thus, the unifactorial nature of this dominant gene seems established. It is important, however, to emphasize that our studies have been limited to one strain of mouse hepatitis which is adapted to, and maintained in, a susceptible line of mice (PRI). It is likely that different results would be obtained with other strains of virus, for such strains have been recovered from different strains of mice, and at least one of these fails to grow in our susceptible mice (7). The ability of the virus to change its tissue specificity was apparent in our first study of its growth in macrophage cultures. Only after a series of passages did the virus destroy parenchymal liver cells. Mosely also showed that there was a rapid change of tissue specificity on tissue culture (8). In the present study the partial adaptation to C_3H mice, whereby it acquired the capacity to kill adult mice after passage through infant C_3H mice, probably reflects a similar trend.

Our original demonstration of the genetically resistant and susceptible macrophages was done with cultures of infant mice (1), and in this we showed that resistant strains of mice yielded cultures which remained resistant for a period of 10 days. The present finding that infant mice from the C_3H-resistant line themselves are actually susceptible but with a delayed incubation period, led us to a reexamination of the original tissue culture results.

Delayed susceptibility in tissue culture was found to mirror the delayed susceptibility of the infant mice, but to be subject to modification by the environment. A difference in the culture media in the original study where 30% serum was used, as contrasted to the 90% serum used in the present study, may also partly explain the results, and should be investigated. It is suggested, then, that macrophages may be separated into three classes; (*a*) the mature genetically resistant, (*b*) the susceptible, and, (*c*) a delayed susceptible when derived from infant-resistant mice.

Increasing resistance to virus infections with age is a well recognized but poorly understood phenomenon (9). It has been demonstrated in chick embryos to equine encephalomyelitis (10). and in mice to vesicular stomatitis (11, 12), and to herpes viruses (13). Recently, Johnson has suggested that there is a cellular basis for the increase with age of the resistance of mice to herpes simplex virus (14). He has shown that virus spreads much more easily in cultures of suckling mouse macrophages than it does in similar cultures of macrophages from adult mice. We have not demonstrated in mouse hepatitis what the nature of the cellular resistance is, but have found that susceptibility, although correlated with the age of the mouse from which it is taken, may be modified by the extent of growth of the culture. Thus, the more rapidly growing cells obtained from younger mice create conditions of greater susceptibility on a mass basis alone, and precautions must be taken to avoid this effect. In our experiments it was not possible to count the number of cells, since we dealt with explants of liver tissue which contained the susceptible macrophages. However, cultures which produced relatively few macrophages were eliminated from all experiments, and only cultures containing roughly comparable numbers of macrophages as judged visually were used. In addition, resistant cells have been routinely obtained from adult (weaned) C_3H mice, and susceptible cells from infant C_3H mice. The exact time of transition to resistance is however difficult to determine but cells from mice of 16 days or more of age have not been found susceptible.

SUMMARY

Adult or weanling C_3H mice were found to be genetically resistant to a strain of mouse hepatitis virus. Infant C_3H mice, however, developed infection and died from mouse hepatitis virus when minimal infectious doses of virus were given to them. There was a delay in the time of death compared to that of the

genetically susceptible strain, and the virus recovered from these mice had increased pathogenicity for C_3H mice.

The ontogeny of resistance to hepatitis in the C_3H mice thus progresses from delayed susceptibility to complete resistance as the age of the host increases. It is reflected in increased resistance of macrophages derived in vitro from liver cultures of infant mice of different ages. This increase in resistance with age was reduced by maintaining the cultures for a longer period of time before inoculation, or by increasing the number of explants in a given culture. Resistant cells were uniformly furnished by mice age 16 days, or more. It is concluded that a process of maturation of resistance of the cells takes place after the mice are born, but that this does not continue under in vitro conditions, and that it may be modified by the environment of the cells.

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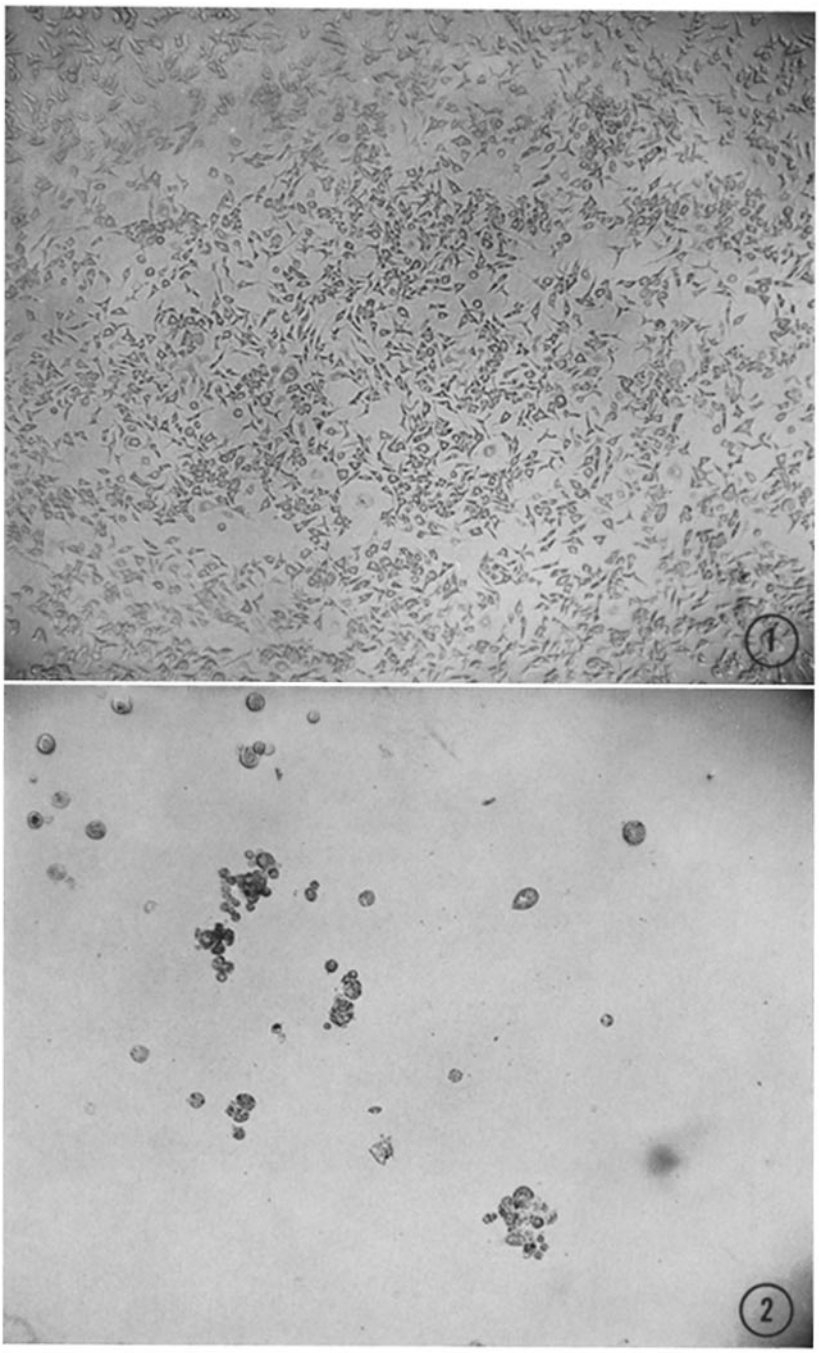
EXPLANATION OF PLATES

PLATE 70

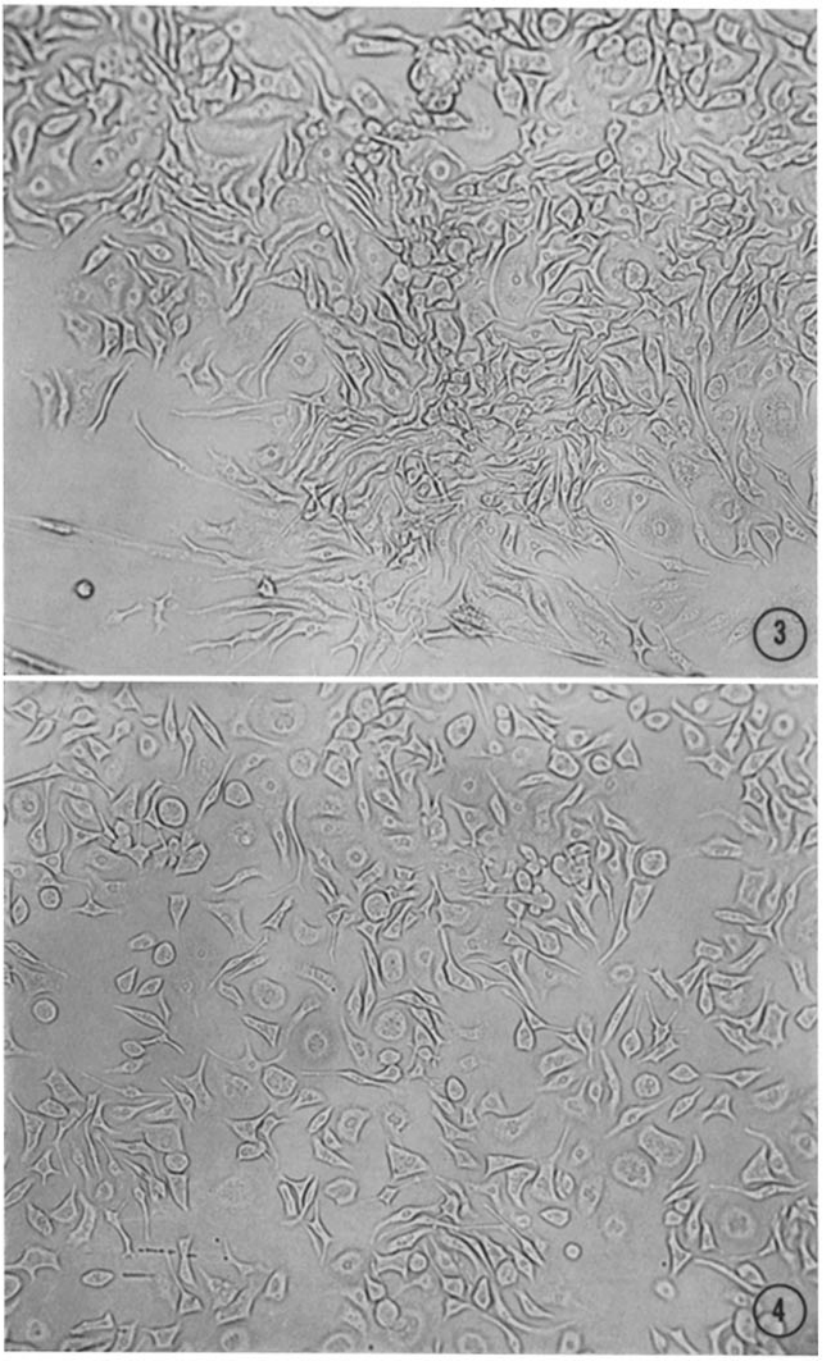
FIGS. 1 and 2. Macrophages from 1-day-old mouse liver.
FIG. 1. Control, 21 days in tissue culture. × 50.
FIG. 2. Infected with MHV 18 days of infection. × 50.



(Gallily et al.: Ontogeny of macrophage resistance to mouse hepatitis)

PLATE 71

FIGS. 3 and 4. Macrophages from 5-day-old mouse liver.
FIG. 3. Control, 25 days in tissue culture. × 100.
FIG. 4. Infected with MHV (22 days of infection). × 100.



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