

ACUTE HEPATITIS ASSOCIATED WITH MOUSE LEUKEMIA

II. ETIOLOGY AND HOST RANGE OF THE CAUSAL AGENT IN MICE

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The etiology of the acute hepatitis which was observed during the passage of mouse leukemia (1) is dealt with in the present paper, together with the biological characteristics of the specific agent. The susceptibility of Princeton mice at various age levels and of different strains of mice is also considered.

Bacteriological Examination

Repeated examination failed to indicate that the pathogenic activity of liver suspensions from infected mice was referable to any agent demonstrable by light microscopy or cultivable in cell-free media. Early in the work particular attention was paid to the detection of *Bacillus piliformis*. In view of the marked difference between the chronic disease originally reported by Tyzzer (2) and the present acute malady, there seemed to be little likelihood of an association with this organism. Though it is not cultivable in artificial media, its appearance is highly distinctive, and it was never observed in liver sections, suspension films, or impression films. Because of the frequent association of leptospira with diseases of the liver, these organisms were also sought in wet mounts and silver-impregnated films, but were never seen.

Bacteria of any sort were rarely present on microscopic or cultural examination. The microscopic picture was unlike that of the occasionally encountered liver abscess in which leukocytes are prominent and many bacteria are seen. Heart infusion-agar plates with and without horse serum enrichment showed no significant or consistent growth of bacteria. From time to time an occasional colony was observed. These were not identified, but it was obvious they were of no etiological consequence. Bacteriologically sterile suspensions were not infrequently obtained from the affected livers.

The Titration of Liver Suspensions

The titer of the causal agent in liver suspensions was determined from the mortality of mice injected with ten-fold dilutions.

The pooled livers from three to five sick mice, killed on the 2nd to the 3rd day after injection, were weighed and ground; and a 10 per cent suspension was prepared in saline. Suc-

cessive dilutions were made from the un sedimented suspension and 0.1 ml. amounts injected intraperitoneally in each of three or five mice. The volume of the inoculum was not considered, in expressing the dilution value.

In an initial experiment, using dilutions from 10^{-2} through 10^{-6} , the end-point of activity was not obtained. One mouse in the 10^{-3} dilution group and one in the 10^{-6} group survived. All of the others died between the 3rd and 7th day. The results of two additional titrations, in which the dilution series was extended, are summarized in Table I. Declining activity with an increasing survival time (the interval between injection and death) occurred through the 10^{-7} dilutions. The 10^{-8} dilutions were inactive. All the surviving mice were killed after 14 days. One mouse in the 10^{-6} group showed marked cirrhosis of the liver at autopsy and one in the 10^{-7} group, numerous surface pits. The

TABLE I
Titer of the Virus in Liver Suspensions

No. of mice injected	Dilution of suspension	No. of mice dying	Average period until death
			<i>days</i>
8	10^{-4}	8	3.6
"	10^{-5}	8	3.7
"	10^{-6}	7	5.1
"	10^{-7}	4	7.0
"	10^{-8}	0	—

livers from two of the mice in the 10^{-7} group and from the five in the 10^{-8} group were all normal. No passages were made from them.

The Sedimentation of Liver Suspensions

The effect of sedimentation on the activity of liver suspensions was determined, first by low, and then by high speed centrifugation.

Spinning a saline suspension of pooled ground livers from infected mice in a horizontal centrifuge at 1000 R.P.M. for 15 minutes (radius 9 cm.), resulted in a bulky sediment and moderately turbid supernatant. In a titration carried out by the intraperitoneal injection of mice, in groups of three, with 0.1 ml. amounts of undiluted supernatant, dilutions 10^{-3} and 10^{-5} , all the animals died within an interval of 6 days. This finding was quite unlike that resulting from the low speed sedimentation of leukemic material. Mice injected with supernatants from centrifuged spleen suspensions very rarely showed any manifestations of leukemia.

Low speed supernatants from other liver suspensions were centrifuged in a small angle centrifuge at a speed of 5000 R.P.M. for 45 minutes (3500 G). This force was sufficient to throw down the bulk of suspended particles as small as the pleuropneumonia-like organisms. Each of two liver supernatants prepared in this way was active, in its undiluted state killing five out of six mice on injection, and in a 10^{-4} dilution killing four out of six.

The supernatants from liver suspensions spun with still greater force also retained their activity. Low speed supernatants were sedimented in a larger angle centrifuge at 8000 R.P.M. (10,000 G) for 45 minutes. The undiluted supernatant (upper third) killed 3 out of 3 mice and the 10^{-4} dilution 2 out of 3. In a later experiment with another liver suspension centrifuged in the same way, the dilution range was extended. The supernatant showed declining activity through the 10^{-7} dilution and was comparable in titer to the same suspension prior to centrifugation. The deaths in the titration of the supernatant were as follows: dilution 10^{-4} 3/3, 10^{-5} 2/3, 10^{-6} 2/3, 10^{-7} 1/3. Sedimentation with this force resulted in a small pellet of sediment, a clear supernatant, and a compact white surface film composed largely of fat droplets.

The Filtration of Liver Suspensions

The filterability of the causal agent was determined by tests of the filtrates from suspensions of 4 different livers, which were passed through Berkefeld V filters.

10 per cent suspensions of weighed and ground livers were prepared in heart infusion bouillon (pH 7.4). They were sedimented at low speed in a horizontal centrifuge (1000 R.P.M. for 15 minutes) and the supernatant fluid removed. About 10 ml. of this, turbid but essentially cell-free, was filtered through short Berkefeld V candles and yielded 2 or 3 ml. of a clear filtrate. Mice, in groups of 3, were injected intraperitoneally with 0.1 ml. amounts of the undiluted filtrates and of 10^{-4} dilutions in saline.

Each of the four filtrates produced hepatitis in the two concentrations employed. Eleven of the twelve mice injected with the undiluted filtrates and nine of those which received the 10^{-4} dilution died on the 3rd to the 5th day. The four normal appearing survivors were killed on the 7th day. At autopsy three showed scattered areas of focal necrosis in the liver and one, from the 10^{-4} group, had healthy looking hepatic tissue.

On the basis of these observations, the causal agent of acute hepatitis of the mouse is regarded as a virus and will be referred to as such.

Protection Tests with Serum from Surviving Mice

As previously noted, a few weanlings showed no signs of illness following the injection of pathogenic liver suspensions by one or another route. Protection tests were carried out with blood serum from ten of these mice, assembled from different experiments.

2 to 3 weeks after injection the ten surviving mice were killed with ether. Throughout this period they had been healthy in appearance and gained weight normally. Prior to death, blood was drawn from the heart into a 1 per cent sodium citrate solution or a 27 mg. per cent heparin solution (0.1 ml. per 1 ml. of blood). Because the surviving mice are infrequent, it was necessary to store the blood in the refrigerator until an amount sufficient for testing was collected. Three different pools of blood were ultimately made. The serum was separated and mixed with an equal volume of a pathogenic liver suspension, diluted 10^{-1} and 10^{-4} . The serum-suspension mixtures were placed in the refrigerator for 1 hour and 0.1 ml. injected intraperitoneally in each of three weanlings.

In the three protection tests, eighteen mice were injected with the serum-liver mixtures. With each of the two dilutions (10^{-1} and 10^{-4}) seven of the nine mice died between the 3rd and the 7th day. The four survivors showed pitting of the liver at autopsy on the 10th day. The number of tests and the dilution range of the suspensions were restricted by the small volume of serum available.

The findings are too limited to be conclusive but they indicate that no significant amount of protective antibody had been produced by the surviving mice.

The artificial immunization of mice was not attempted but five, that had recovered after injection and held under observation for several weeks, were reinjected with a pathogenic liver suspension.

Five weanlings which had recovered after the injection of a low titered virus suspension, were challenged a month later by the intraperitoneal injection of 0.1 ml. of a 10 per cent liver suspension. Three of these mice died on the 3rd day after injection. Two were alive and normal on the 10th day. They were killed at this time and, on autopsy, showed the characteristic pitted livers. When reinjected, the five mice had weighed 18 to 23 gm. As will be noted in a later section, Princeton mice in this weight range do not develop as pronounced hepatitis as weanlings.

Hemagglutination Tests with Liver Suspensions

Attempts were made to learn whether the virus of mouse hepatitis agglutinated red blood cells from normal mice.

In these experiments, 0.1 ml. of a 2 per cent suspension of washed RBC in saline was added to 0.9 ml. of the solution to be tested. After thorough mixing the tubes were placed in the incubator at 37°C. for 1 hour and in the refrigerator overnight. The use of supernatants from liver suspensions centrifuged at 5000 r.p.m. was hampered by the presence of hemolyzing substances. The supernatants of both normal and pathologic liver suspensions (10 per cent in saline) tended to produce hemolysis. In some instances complete clearing occurred after 1 hour at 37°C. In the absence of hemolysis, there was no agglutination. Two out of three Berkefeld V filtrates which contained the virus of acute hepatitis were not hemolytic but did produce a slow agglutination of the RBC, detectable after overnight refrigeration. At present this finding can be regarded as no more than suggestive of a specific result.

Distribution of the Virus in Mice

Tests were made for the presence of virus in organs other than the liver and in certain of the body fluids.

The materials to be examined were removed from infected mice killed in the acute stage of hepatitis. At least two separate experiments were conducted with each of the inocula, which were injected intraperitoneally in 0.1 ml. amounts. The numbers of deaths which occurred were as follows: 10 per cent spleen suspension in saline, 7/9; 10 per cent kidney suspension in saline, 7/9; citrated heart's blood, 5/6; urine, 5/8; and intestinal contents, 6/6. The latter suspensions were made from the upper third of the intestine, the fluid being aspirated to 1.0 ml. of saline and 1000 units of penicillin added. The mice that survived after the 7th or 8th day were killed and autopsied. All of them showed definite but reduced manifestations of liver necrosis. In no case was there any significant departure from the hepatitis produced by liver suspensions save that the death rate, in general, was somewhat decreased.

Sensitivity of the Virus to Antibiotics

Early in the work, the effect of penicillin on the activity of the virus was determined, as it was desirable to add an antibiotic to liver suspensions contaminated with bacteria.

Two groups of five mice were injected with 0.1 ml. of 10 per cent liver suspensions containing 1000 units of potassium penicillin G per ml. and one group with a similar mixture that had stood for 7 days in the refrigerator. The penicillin had no demonstrable action on the virus. All the injected mice were dead by the 7th day and showed typical manifestations of hepatitis.

At a later time, the need for eliminating pleuropneumonia-like organisms from certain liver suspensions arose. These bacteria are resistant to penicillin but are inactivated in cultures by aureomycin in a concentration as low as 2.5 mg. per ml. of medium.

Five weanlings were injected intraperitoneally with 0.1 ml. of a 10 per cent liver suspension containing 2.5 mg. of aureomycin per ml. The suspension was then placed in the refrigerator for 4 days and five additional mice were similarly injected with the same dose. The activity of the virus was not affected by the antibiotic in the concentration employed. Nine of the ten mice died on the 3rd or 4th day after injection. One mouse, which was killed on the 5th day, showed diffuse necrosis of the liver at autopsy.

Following the observation of Gledhill and Andrewes (3) that the mouse hepatitis described by them was prevented by the prior injection of terramycin, this antibiotic was also tested.

In the first experiment each of five mice was injected subcutaneously with 0.1 ml. of saline containing 2 mg. of terramycin. Higher concentrations were found to be extremely irritating on injection. The subsequent intraperitoneal injection of a pathogenic liver suspension resulted in the prompt death of all the mice. In the second test the mice were injected twice daily for 3 days with the same dosage and then challenged by intraperitoneal injection of the virus. All of these mice also died. In the third test, mice were presented with terramycin in their drinking water (100 mg. in 100 ml.) for 2 days before injection. Challenged in the usual way, these likewise died.

As employed in these experiments, terramycin had no effect on the activity of the virus.

Inoculation of the Virus into Embryonated Eggs

An attempt was made to cultivate the virus in embryonated hens' eggs. The inocula used in these experiments were 10 per cent liver suspensions, Berkefeld V filtrates, and supernatants which contained the agent in an active state.

Most of the inoculations were made on the chorioallantoic membrane of 10-day-old eggs. In some instances, 1000 units of penicillin was added to the inocula. The eggs were incubated at 37°C. and opened at intervals of 3 to 10 days. The membranes were removed to a Petri dish and examined with a dissecting microscope. For serial transfer and the injection of mice, approximately 10 per cent suspensions were made in saline with the aid of a glass tissue grinder. In one experiment, seven successive transfers were carried out at weekly intervals. A few inoculations were also made into the allantoic fluid of 10-day-old eggs and the yolk sac of 5-day-old eggs.

Save for an occasional bacterial contamination, the inoculations had no effect on the development of the embryo. The supporting membranes showed no consistent structural al-

teration. Mice injected intraperitoneally with membrane suspensions and fluids remained normal during an observation period of 10 to 14 days. At autopsy there was no demonstrable liver involvement.

It was evident from these observations that the hepatitis virus had not been cultivated in embryonated hens' eggs nor was it capable of survival in pathogenic form.

Viability of the Virus on Storage at Low Temperature

Preliminary tests had indicated that the activity of 10 per cent liver suspensions in saline was retained on storage in the refrigerator at 35°F. for intervals up to 10 days. A few additional observations were made on the viability of the virus after longer periods of storage.

In these experiments no attempt was made to determine the actual titer of the virus. A 10 per cent liver suspension held for 50 days at 35°F. resulted in the death of five out of five weanlings on the 4th day after intraperitoneal injection. A second suspension stored for 175 days at the same temperature proved inactive. The five mice injected with it showed no signs of illness and at autopsy on the 10th day their livers were normal in appearance. A third suspension maintained for 135 days in a frozen state at -5°F. was also tested. The five mice that were injected died on the 4th or the 5th day. The same suspension was inactive when tested after 305 days of storage. The five injected mice showed no evidence of hepatitis.

The Examination of Sections and Films for Inclusion and Elementary Bodies

Acidophilic intranuclear inclusions in the hepatic cells of supposedly normal mice were first observed in 1932 by Findlay (4) in adults of a strain of mice known as Clacton. They were not detected in seven other strains. There was some evidence that a virus of low pathogenicity was associated with their presence. Similar inclusions were later observed in normal mice from other colonies by Thompson (5) in 1934, Olitsky and Casals (6) in 1945, and Pavilanis and Lépine (7) in 1949. The etiology of these inclusions is still obscure.

Gledhill and Andrewes (3) in 1951 described acidophilic cytoplasmic inclusions in parenchymal cells from the livers of weanlings infected with their mouse hepatitis virus. These inclusions were observed as early as the 3rd day after injection. They enlarged rapidly and became difficult to distinguish from necrotic material. Acidophilic intranuclear inclusions were also noted infrequently.

Many Giemsa-stained liver sections from Princeton weanlings and a few from adults in various stages of acute hepatitis were examined. Owing to the rapid extension of the necrosis, especially in weanlings, large areas normally occupied by hepatic cells were soon filled with acidophilic debris which included some spherical masses. There was no sign, however, of inclusions in either the cytoplasm or the nucleus of the intact hepatic cells bordering and within these areas of necrosis, or of the parenchymal cells in uninvolved areas.

Impression films from the cut surfaces of diffusely necrotic livers and films from the supernatants of sedimented suspensions were also made. These preparations were impregnated with silver by the Morosow method. Microscopic

examination failed to reveal particles sufficiently uniform in size and shape to be regarded as elementary bodies.

The Susceptibility of Princeton Weanlings to Acute Hepatitis

Princeton weanlings (3 to 4 weeks old) injected intraperitoneally with the standard inoculum (0.1 ml. of a 10 per cent liver suspension) showed nearly uniform susceptibility to acute hepatitis. In addition to the 100 mice held throughout the entire course of the disease, a second passage series numbering 100 mice or more was maintained for the preparation of liver suspensions. In this series several mice from each passage were killed in the acute stage of hepatitis, generally on the 2nd or 3rd day after injection. As indicated by the presence of liver lesions at autopsy, the morbidity rate of the two injection series was 100 per cent. The mortality rate was slightly less, estimated as 98

TABLE II
The Response to the Virus of Acute Hepatitis in Older Princeton Mice Swiss Weanlings, and BSVS Weanlings

Strain of mice	No. of mice injected	No. of mice dying	Per-centage mortality	Average period until death	No. of survivors with liver lesions	Per-centage morbidity	No. of survivors with normal liver
				<i>days</i>			
Young Princeton adults.....	36	18	50	4.3	14	88	4
Old Princeton breeders.....	35	25	71	4.5	7	91	3
Swiss weanlings.....	50	2	4	4.5	1	6	47
BSVS weanlings.....	26	3	12	6.6	6	34	17

per cent. Three mice in the second series were alive on the 10th day and normal in appearance but showed liver involvement on postmortem examination.

In these experiments and those that follow, the injections were made with freshly prepared suspensions of liver from successive passages of the virus in Princeton weanlings. It was considered that these suspensions gave more uniform results than ones that had stood for some time in the refrigerator or in a frozen state. In the tests with older mice and those of other strains, the suspensions were also injected into Princeton weanlings. All the mice that died were autopsied unless postmortem changes were too far advanced for examination.

The Susceptibility of Older Princeton Mice to Acute Hepatitis

Two additional age groups of Princeton mice were similarly tested for their susceptibility to acute hepatitis.

Young Adults.—The first group was composed of 36 young adults of both sexes, 8 to 15 weeks old (weighting 25 to 35 gm.).

In comparison with weanlings, as indicated in Table II, these mice showed a marked drop in the mortality rate, from 98 to 50 per cent, and an appreciable decrease in the morbidity

rate, from 100 to 88 per cent. The average interval between injection and death was somewhat greater than with weanlings, 4.3 days, as compared with 3.5 days. Eighteen of the 36 mice were normal on the 10th to the 12th day after injection. At autopsy the livers of four were also normal, while twelve showed surface pits and two, foci. The pooled livers from fifteen of the survivors, in three different lots, were innocuous on intraperitoneal injection in Princeton weanlings.

Old Breeders.—The second group was composed of 35 discarded breeders, 9 to 12 months old. The males weighed between 32 and 40 gm. and the females 40 and 58 gm.

The mortality and morbidity rates are given in Table II. Both rates were less than the corresponding ones in weanling mice but the mortality rate was appreciably greater than that for the young adults. There was again a noticeable increase in the average number of days to death. Six of the ten survivors, at autopsy on the 10th day, showed pitted livers and one, focal lesions. There was no reaction in weanlings injected intraperitoneally with pooled livers from six of the survivors.

The Susceptibility of Weanlings from other Strains of Mice

The susceptibility of weanling mice from four additional strains was also determined. Both males and females were tested, in groups of five, using the standard inoculum. The results with two of these strains, Swiss and Webster's BSVS, are summarized in Table II. Sufficient numbers of the C albino and Bagg mice were not available, at the time, to provide adequate data, and these mice are considered separately.

Swiss Mice.—The fifty Swiss mice (10 to 12 gm. in weight) were from a random bred colony maintained at the Rockefeller Institute in New York.

After intraperitoneal injection, these mice were held under observation for 10 to 14 days. The mortality rate was 4.0 per cent and the morbidity rate 6.0 per cent. One other mouse, killed on the 14th day, showed a pitted liver. The livers of the remaining 47 mice were normal at autopsy. Pooled liver suspensions from seven of the ten groups of survivors were injected intraperitoneally in Princeton weanlings, with somewhat irregular results. The suspension from the group which included the mouse with a pitted liver killed four out of five weanlings and that from one other group with normal livers killed one out of five. The suspensions from five of the groups were innocuous.

BSVS Mice.—The BSVS mice were from the inbred strain originally developed by Dr. Leslie Webster, from the Rockefeller Institute strain.

This inbred colony is now maintained at the Institute in New York under the direction of Dr. Howard Schneider. These mice apparently gain weight somewhat more rapidly than do weanlings of the Princeton strain. In this respect the BSVS mice resemble those from the parent colony, the rapid growth of which is recognized. In the first lot received from the BSVS colony were a number of mice weighing 16 to 19 gm. but the same age as the Princeton weanlings. These mice were included in the group listed in Table II. The mortality rate (12 per cent) and the morbidity rate (34 per cent) were much reduced in comparison with the rates of Princeton weanlings. The average interval to death (6.6 days) was appreciably in-

creased. The 23 survivors were killed on the 10th to the 12th day after injection. Seventeen of these mice were normal, four showed pitted livers, and two a focal reaction. On intraperitoneal injection in Princeton weanlings, active virus was demonstrable in the pooled normal livers from nine of the surviving BSVS mice and in the pitted livers from four.

C Albino Mice.—The C albinos were obtained through Dr. E. Stanfield Rogers from the inbred colony of Dr. Peyton Rous.

Ten weanlings, five males and five females, were tested. Two mice, both females, died on the 6th day. All of the eight survivors, though normal in appearance, showed pitted livers when killed on the 10th day. Despite the small number of mice, the high morbidity rate (100 per cent) is of interest in view of the low mortality rate (20 per cent). The pooled livers from the five surviving males killed two out of five Princeton weanlings on intraperitoneal injection.

Bagg Mice.—The Bagg mice were originally from an inbred colony maintained by Dr. Clara Lynch at the Rockefeller Institute.

The breeding of the Bagg colony was discontinued in 1951. At this time, a few adults were held over in our laboratory and random bred for a period of 6 months. As before, ten weanlings, five males and five females, were tested. All these mice survived and at autopsy, on the 10th day after injection, showed normal livers. A pooled suspension of the livers from the five males, killed four out of five Princeton weanlings on intraperitoneal injection. The single survivor showed pitting of the liver on postmortem examination.

DISCUSSION

As previously noted, there is a close similarity between the acute hepatitis of Princeton weanlings and the mouse hepatitis described by Gledhill and Andrewes (3). There would appear to be differences, however, among the viruses causing these diseases in respect to titer, size, and their sensitivity to aureomycin and terramycin.

Weanlings of the Princeton strain were almost uniformly susceptible to acute hepatitis virus in high concentration. The mortality and morbidity rates were nearly equal and close to 100 per cent. With a low concentration the results were more variable and commonly the mortality rate was appreciably less than the morbidity rate. The seemingly normal survivors often showed reparative changes in the liver, indicative of previous injury by the virus, although in most instances the causal agent was not demonstrable on passage. It was apparent that Princeton mice of this age possessed a limited means of defense. There was no experimental evidence, however, that recovery was associated with the production of a protective antibody.

Older Princeton mice were significantly less susceptible than weanlings. The increased ability to cope with the disease was particularly noticeable in young adults. In these mice the mortality rate declined nearly 50 per cent and the morbidity rate approximately 10 per cent. With old breeders there was an increase in their rates, although both were appreciably less than in weanlings.

The VS mice tested by Gledhill and Andrewes (3) showed a much greater variation in susceptibility with age. The mortality rates reported by them for weanlings and somewhat older mice were 96 and 16 per cent, respectively.

Princeton weanlings were by far the most susceptible of the five mouse strains that were tested. As indicated by the mortality and morbidity rates, the Swiss and BSVS weanlings were even less susceptible than young adults of the Princeton strain. With both of these strains there was a marked decrease in the number of survivors which showed any indication of liver involvement at autopsy. The low mortality rate (12 per cent) of the BSVS mice was particularly significant. The VS mice which showed an incidence of 96 per cent in the experiments of Gledhill and Andrewes (3) were of the same ancestry. The susceptibility of the few Bagg and C albino mice that were tested was also low. With three of these strains a condition was encountered which was not observed with Princeton weanlings. A few normal survivors continued to carry active virus in the liver, without detectable involvement, for a period up to 2 weeks after injection.

The observations on strain susceptibility indicated an additional point of departure between the two hepatitis viruses. The greater activity of the present virus, in respect to titer, might explain some of the previously noted differences but would not account for the markedly dissimilar behavior in Webster's selected and inbred mouse strain. Until additional comparisons are made, however, the actual relation of these two viruses cannot be defined.

SUMMARY

The etiological agent of the acute hepatitis encountered in weanling mice of the Princeton strain is a virus found in the liver, spleen, kidneys, heart's blood, urine, and intestinal contents of experimentally infected animals. Older Princeton mice and weanlings from four other strains (Swiss, Webster's BSVS, C albino, and Bagg) proved to be less susceptible than Princeton weanlings.

The virus was demonstrable in Berkefeld V filtrates of liver suspensions and in supernatants after centrifugation at 10,000 *G*. In whole liver suspensions it was detectable in decreasing amounts through a dilution of 10^{-7} .

Aureomycin and terramycin had no detectable effect on the activity of the virus in weanlings.

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