AN INFECTIOUS HEPATITIS OF UNDETERMINED ORIGIN IN MICE*

II. Characteristics of the Infective Agent

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The discovery of an agent which produces transmissible hepatitis and ascites in mice has been described in a preliminary report (1) and in the preceding paper. It seems probable that it was carried by the strain of mice under study. The disease resembles in certain respects many known diseases in mice. For this reason, it seemed important to compare this agent point by point with a number of the other known pathogenic agents occurring in the O'Grady strain of mice. Evidence will be presented that the agent differs from the more common bacteria, leptospira, protozoa, rickettsia, and viruses known to produce disease in mice. It is believed that the malady it causes has not been described previously. For convenience of discussion the agent will be called ascites hepatitis agent (AHA).

Agents Studied

Ascites Hepatitis Agent (AHA).—The strain of AHA first procured by serial passage in mice, after an original inoculation with an extract of the liver of a patient (C.B.), was used in all the studies described below. Infectious organ extracts were prepared and stored as described in the preceding paper (2). In many experiments ascitic fluid was also utilized.

Lymphocytic Choriomeningitis Virus (LCM).—Two strains of LCM virus were compared with AHA. One was strain Armstrong E 350-6 obtained from the National Type Culture Collection. The Armstrong strain which had been carried through fourteen intraperitoneal passages in mice was obtained from Dr. Wallace Rowe at the Naval Medical Research Institute. Infective extracts of this virus were stored in tightly stoppered pyrex tubes in the dry ice chest.

Mouse Hepatitis Virus (MHV).—An ampoule containing the lyophilized mouse hepatitis virus (MHV) described by Gledhill and Andrewes (3–5) was provided by Dr. C. H. Andrewes. This strain of virus (MHV 5841/58) was maintained by serial, weekly, intraperitoneal passage of mouse liver extracts in 2 to 4 gm. suckling mice of the O'Grady or A strains and infected organs and saline extracts of combined livers, spleens, and kidneys were stored in the dry ice chest.

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EXPERIMENTAL

Characteristics of AHA

The Virulence of AHA.—The virulence of AHA was difficult to quantitate because of the generally mild character of the infection and its low mortality. On many occasions serial tenfold dilutions of organ extracts or ascitic fluid in 0.85 per cent saline or in trypticase soy broth were inoculated intraperitoneally into mice. The infectivity titer of fresh 10 per cent organ extracts was usually 10^{-2} and on a few occasions 10^{-3}. Dilutions greater than this were never infectious. No detectable increase in virulence occurred during 14 serial monthly passages after the disease was first recognized.

TABLE I

The Effect of Storage at −70°C. on the Infectivity of Ascites Virus of Mice (AHA)

Virus preservation	No. of experiments	Total No. of mice	Signs of infection in mice			
			Ascites		Hepatitis	
			No.	Per cent	No.	Per cent
Used fresh	6	130	60	46	109	84
CO_2 chest, 1 day–2 mos.	6	141	44	31	73	52
CO_2 chest, 2 mos.–5 mos.	11	433	74	17	209	48
Total.....	23	704	178	25	391	56

The Stability of AHA.—A single quick freezing of AHA suspensions in a dry ice–alcohol mixture with thawing on the subsequent day usually resulted in a tenfold fall in infectivity titer. Quick freezing and thawing ten times completely destroyed its infectivity. The effects of storing the agent in a dry ice chest of −70°C. for various periods are shown in Table I. It will be seen that the average incidence of hepatitis and ascites decreased markedly within a few months. Hepatitis with ascites occurred in 46 per cent of inoculated mice when freshly harvested virus was used, in 31 per cent after storage in a dry ice chest for one day to 2 months, and in 17 per cent after 2 to 5 months' storage. The incidence of hepatitis without ascites in the inoculated mice was 84 per cent, 52 per cent, and 48 per cent respectively. Ascites was never observed in the absence of hepatitis.

The infectivity of some virus preparations resisted storage for over 6 months in the dry ice chest at −70°C. In several experiments aliquots of virus were stored at several temperatures for various times. These aliquots were then compared for infectivity, as summarized in Table II. Although the number of animals inoculated was small, it seemed from the findings that storage in an electric deep freeze at about −12° was less effective than in the dry ice chest. This was surprising since some preparations of the virus remained infective

for at least 4 months at 4°C. Infectivity was practically destroyed by heating virus suspensions at 60°C. or more for 1 hour, and was greatly decreased by 56°C. for 1 hour.

Five attempts were made to preserve the virus by drying it in the frozen state. In no instance did this lyophilized material result in unequivocal infection.

Hydrochloric acid (4 N) was added to some aliquots of infective organ extracts in amounts sufficient to bring the pH to 1, and sodium carbonate solution to others to make the pH 9. After 4 hours at 4°C. the normal pH, 6–7, was reconstituted and mice were inoculated. Both treatments resulted in decreased infectivity, but not in complete loss.

TABLE II

Effect of Temperature on Infectivity of AHA

No. of experiments	Time	Temperature					
		−70°C.	−12°C.	+4°C.	+56°C.	+60–65°C.	+100°C.
3	1 hr.	—	—		2/6/13*	0/2/7	0/3/13
2	3 days	3/ 3/ 3	—	10/10/10	—	—	—
1	3 wks.	1/ 2/ 2	—	3/ 3/ 3	—	—	—
3	2–3 mos.	13/12/17	0/1/ 9	5/ 5/ 7	—	—	—
4	4–6 "	5/ 8/15	2/3/11	3/ 3/ 4	—	—	—
Total		22/25/37	2/4/20	21/21/24			

* The first number represents the mice with ascites; the second the mice with hepatosplenomegaly, and the third the total inoculated.

Size of AHA.—Though the actual size of AHA has not been determined, it would seem to be considerably larger than most viruses. Filtration through Whatman paper 2, which held back most of the cells, did not seem to diminish the infectivity of organ extracts or of ascitic fluid, but the filtration of extracts or ascitic fluid through a single Seitz pad removed infectivity as several such tests made plain. Only the sediment was infective after centrifugation of these materials at 13,000 R.P.M. for 30 minutes. Centrifugation at only 2500 R.P.M. for 20 minutes yielded equivocal findings but when it was done at this speed for 1 hour the infectivity was concentrated in the sediment. However, these findings should be viewed in the light of the fact that the materials were of such low infectivity. Even the relatively small pneumonia virus of mice (PVM) (6) will give similar results when strains of low titer are tested (7).

Failure of AHA to Agglutinate Erythrocytes.—Numerous fruitless attempts were made to produce hemagglutination of erythrocytes with AHA. Infective ascitic fluid and organ *brei* were compared with normal tissue *brei*, fresh and after heating at 56° or 65° for 1 hour. The washed erythrocytes of mice, hamsters, rabbits, chickens, and normal human beings of blood group O were all

tested, and the tests were made both at room temperature and 4°C. and were read at various intervals for 12 hours. In no case was hemagglutination obtained in higher titer than with normal tissue extracts. If AHA can cause the agglutination of erythrocytes it must be with cells from other species or by other techniques than those utilized.

TABLE III

Effect of Antimicrobial Agents on AHA Infection in Mice

Drug	Daily Dose	Route	Time in days	No. of Expts.	Results	
					Treated	Controls
Sulfadiazine.....	10 mg.	Per os	0–28	1	3/ 4/5*	2/ 2/ 6
Penicillin.....	100 units	Subcutaneous	0–1	1	5/ 5/ 5	
"	" "	"	0–7	1	5/ 4/ 5	5/ 5/ 5
"	" "	"	7–14	1	5/ 5/ 5	
"	" "	"	14–21	1	4/ 4/ 5	
Penicillin	*Ad libitum*	Per os	0–28	2	9/13/16	10/12/12
"	100 units	Subcutaneous	14–28	2	8/10/12	6/ 8/10
"	" "	Intraperitoneal	0–28	2	0/ 0/ 8	5/ 6/ 6
"	*Ad libitum*	Per os	0–28	3	9/13/19	12/14/14
Aureomycin.....	1 mg.	Subcutaneous	24–32	1	2/ 3/ 3	1/ 4/ 6
Terramycin.....	25 mg.	Per os	0–28	2	5/ 5/ 8	2/ 2/ 6
Streptomycin....	500 units	Subcutaneous	0–28	2	3/ 4/ 8	3/ 7/ 8

* Scoring as in Table II.

The Effect of Antimicrobial Agents on AHA Infection

Several antimicrobial agents were tested separately for their effects on AHA infection.—

Sodium sulfadiazine was given to inoculated mice as a 0.1 per cent solution in the drinking water for 1 week after inoculation but then discontinued because of its toxicity. The water supplied these mice did not contain any urethane. The other drugs tested were given throughout the whole month from the day of virus inoculation until the time of sacrifice. Some mice were provided *ad libitum* with an aqueous solution of penicillin G containing 50 units per cc. Others were given penicillin G subcutaneously in a dose of 100 units/mouse/day in one or two divided injections. Aureomycin hydrochloride, 1 mg./mouse once daily, was injected subcutaneously in a saline solution containing 5 per cent gum acacia. Other mice received a 0.25 per cent aqueous solution of terramycin to drink. Streptomycin, 50 units/mouse/day, was injected in 2 divided doses into yet other mice.

The results of these experiments are summarized in Table III. It will be seen that none of the drugs tested seemed to have any inhibiting influence

upon the infection, with the possible exception of penicillin. However, ascitic fluid taken from mice treated with penicillin subcutaneously for 12 days prior to paracentesis proved infective on test.

Cortisone, 0.25 mg./mouse/day, injected subcutaneously, did not affect the infection rate when given during the first 2 or the last 2 weeks of the incubation period. It may have had some effect to lessen the ascites and hepatosplenomegaly when given for 4 to 11 days after ascites appeared.

Tests for the Presence in the Blood of Protective Antibodies against AHA

The absence of immunity in mice convalescent from the hepatitis to reinfection with AHA has been reported in the preceding paper. Furthermore,

TABLE IV

Attempts to Neutralize AHA Infection with Specific Sera

Serum	Titer of extract		
	10^{-0}	10^{-1}	10^{-2}
None	3/5/6*	1/2/6	0/0/6
Normal mouse pool	1/1/2	1/1/3	0/1/3
Convalescent mouse pool	2/2/3	0/1/3	0/0/3
Normal rabbit	1/2/3	2/2/3	0/0/3
Anti-mouse rabbit	2/3/3	2/3/3	0/1/3
Anti-AHA rabbit	2/3/3	0/1/3	0/2/3

* The results are recorded as explained under Table II.

it was found that blood collected during the ascitic phase of the disease and extracts of the organs, as obtained up to 6 months after inoculation, were both infective. Nevertheless attempts were made to neutralize AHA with the sera of convalescent mice or of rabbits injected with the agent.

Blood from normal mice and from mice 1 to 2 months after recovery from ascites was collected, pooled, and the serum separated. Normal rabbit sera and the serum from 7 rabbits injected with extracts of livers, spleens, and kidneys from mice that had come down with the disease were also collected. Four of the rabbits had been given 1 cc. of a 10 per cent extract of infective organs intravenously and two had been injected subcutaneouly with a mixture consisting of about 0.2 cc. each of such an organ extract and falba, and 0.1 cc. of mineral oil. An additional rabbit had received 9 intravenous injections over a 3 week period, altogether 18 cc. of a 10 per cent extract of liver-spleen-kidney *brei* from normal mice.

In all these instances the serum tested was obtained about 1 month after the last injection. Tenfold dilutions of 10 per cent AHA extracts were mixed with equal quantities of the undiluted sera, and allowed to stand for 12 hours at 4°C. Normal mice were then inoculated intraperitoneally with 0.2 of the mixtures.

The results are shown in Table IV. It will be seen that none of the supposedly immune sera effected any definite neutralization of the agent. The numbers of mice employed were too small for quantitative interpretation,

but the presence of mice with ascites and hepatitis in the groups given "immune" serum mixtures, excludes the presence of large quantities of protective antibodies. However, complement-fixing antibodies were found to be present in AHA convalescent sera as will be described below.

Attempts to Identify AHA

Among the many diseases indigenous in mice, only a few produce a pathological picture closely resembling that of the disease we have described. Extensive and intensive efforts were made to identify AHA infection, with some one of the maladies already known. The results of these efforts will now be reported.

The Microscopic Examination of Tissues.—Hematoxylin and eosin-stained sections of the liver, spleen, brain, kidney, lung, heart, and ascitic fluid sediment failed to reveal evidence of bacteria, fungi, protozoa, or inclusion bodies. Giemsa-stained slides of liver, brain, spleen, and ascitic fluid sediment were likewise negative. No intranuclear or intracytoplasmic inclusion bodies were ever noted in our laboratory. Gram stains of the tissues showed no bacteria. However, Dr. Anthony Morris (8) of the Army Medical School has demonstrated on several occasions with Giemsa stain the presence of pink-staining pleomorphic inclusion bodies in the nucleus or cytoplasm of occasional cells in ascitic fluid sediment and splenic pulp from a strain of mice infected with AHA. The identity of these bodies is uncertain, as is their relationship to the disease under study. It has been considered that they may represent a protozoan.

Bacteriological Cultures of the Extracts Containing AHA.—Cultures of ascitic fluid and organ extracts from mice infected with AHA were done repeatedly. As routine, Brewer's thioglycollate medium and trypticase soy agar plates containing 10 per cent human blood were inoculated with these materials. Plates were incubated both aerobically and under anaerobic conditions. Infective ascitic fluid usually proved to be sterile but the organ *brei* always was found to contain a few miscellaneous bacteria whenever a large inoculum was cultured. Staphylococci, diphtheroids, or *Bacillus subtilis* were usually found but their presence seemed unrelated to the infection. The intestinal flora from the large bowels of infected mice was cultured on blood agar plates and desoxycholate citrate agar (Baltimore Biological Laboratory). No relation was found between the species of organisms thus cultured and the susceptibility of the mice to AHA. On several occasions cultures of the organ extracts or intestine yielded *Salmonella typhimurium.* These infected preparations were not used for passage, and no epizootic due to that bacterium developed in the mice.

The signs typical of infection with pleuropneumonia-like organisms, namely rolling and polyarthritis (9–11), were never observed in our mice. Special

cultures for pleuropneumonia-like organisms were made on agar plates enriched with 20 per cent human ascitic fluid (12). No strains of this organism were recovered from AHA infected mice, even though the media employed were found on test to sustain the growth of 5 typical strains of this organism obtained through the courtesy of Dr. Harry Morton of the University of Pennsylvania. It was concluded that the infection was not caused by any bacterium known to be pathogenic for mice.

Comparison of AHA with Leptospira.—The infection of mice with leptospira has been reported (13–15) but the resulting changes are not like those of the disease we have described. Nevertheless, a search, which proved fruitless, was made with the darkfield microscope for these organisms in infective extracts and ascitic fluid. Cultures of such materials proved sterile on the medium recommended by Gardner (16) for growing leptospira. In the pooled sera from normal mice or from acutely infected or convalescent mice, Dr. Paul Beeson found no agglutinins against 8 strains of leptospira (*L. ballum*, *L. canicola*, *L. grippotyphosa*, *L. hebdomadis*-T., *L. icterohemorrhagiae*, *L. sackoebing*, *L. pomona*, and *L. mitis*).

It is concluded that AHA is not one of these leptospira.

Comparison of AHA with Protozoa.—Infection of mice with encephalitozoon or toxoplasma may result in a disease generally resembling that due to AHA (17, 18). However, Giemsa-stained sections from the brains of mice with typical AHA infection did not show the curved rods in round cyst-like accumulations, within cells or at the margin of inflammatory lesions, which are typical of encephalitozoon infections in mice. Also brain sections from AHA-infected mice showed no meningoencephalitis (which is present in over 90 per cent of mice infected with encephalitozoon), and none of the extracellular parasites typically seen in toxoplasmosis. Failure to infect rabbits with AHA further differentiates it from encephalitozoon. Toxoplasma, unlike AHA, does not survive storage with dry ice. As stated previously, inclusion bodies possibly protozoan have been demonstrated in some mice infected with AHA by Dr. Anthony Morris (8) but the relation of these to the disease is not certain.

Comparison of AHA with Rickettsia.—No disease in mice due to rickettsia has been described which produces the obvious signs or pathological features of AHA infection. Yet the possibility that AHA is an agent in this group of microorganisms has not been excluded. However, this seems unlikely since no rickettsia have been seen after appropriate staining of infected material, nor has infection been established in the yolk sac of embryonated hens' eggs (2). Serological tests to exclude the known rickettsia were not made.

Comparison of AHA with the Known Viruses of Mice.—A number of viruses may be carried by mice. AHA differs strikingly from some of these, namely PVM (19), GDVII (20), and ectromelia (21, 22), in its strict viscerotropism and its failure to agglutinate erythrocytes. Members of the psittacosis group

seem to be further excluded by the general absence of intracellular inclusion bodies, the failure to infect embryonated eggs, and by the fact that the sera from 6 rabbits immunized against AHA failed to fix complement with lygranum antigen (obtained from E. R. Squibb and Company).

Various workers have reported the occurrence of hepatic lesions in otherwise healthy mice. Olitsky and Casals (23) described infiltration and necrosis, and acidophilic intranuclear inclusion bodies, in the livers of supposedly normal mice of the Rockefeller Institute strain, especially with increasing age. Pavilanis and Lepine (24), Findlay (25), Nicolau and Ruge (26), and Gledhill and Andrewes (3) have described similar inclusion bodies in the livers of mice of other strains. The agent responsible for these bodies was thought to be a virus, and in some instances the condition characterized by them seemed to be transmissible, but in no case did obvious signs of illness result. Careful search for inclusions in the appropriately stained liver cells of mice infected with AHA failed to disclose any like those described in the literature.

Comparison of AHA with "Mouse Hepatitis Virus" (MHV) of Gledhill and Andrewes.—Gledhill and Andrewes (3–5) described a transmissible hepatitis in suckling mice that proves fatal. A specimen of this agent was obtained from Dr. Andrewes and its effects compared with those of AHA in several strains of mice.

These agents caused focal liver necrosis and death within 6 to 10 days in nearly all 2 to 4 gm. mice of our strains. In similar mice, AHA caused typical non-fatal disease with an incubation period of about 2 weeks. Infection with MHV, unlike AHA infection, resulted in livers covered with light spots which later coalesced to involve nearly the whole liver. There was no ascites. However, the hepatic changes in the two infections were indistinguishable histologically. As in the case of AHA, chronic carriers of MHV have been reported, and mice convalescent from MHV infection developed no protective circulating antibodies, or these only in low titer. It was impossible to test convalescent immunity to MHV infection since weanling mice of the strains we employed proved naturally resistant. Since mice of all ages are susceptible to AHA infection it was decided to challenge MHV convalescent mice with AHA. It was found that 5 gm. mice which had survived infection with MHV were as susceptible as normal animals to infection with AHA. Also, weanling mice inoculated with infectious MHV proved susceptible to subsequent infection with AHA. Conversely, litters of mice convalescent from AHA infection were susceptible to MHV.

These findings are not surprising since AHA infection does not result in homologous immunity (2) and MHV infection produces protective antibody only in low titer (3). It seems evident that no relationship between these two agents can be demonstrated on immunological grounds. Moreover differences in their size, the incubation periods of the diseases they produce, the course of their diseases, and a differing age susceptibility of inoculated mice serve thus far to distinguish them.

Comparison of AHA with the Acute Hepatitis Virus of Nelson.—The viral hepatitis of mice described by Nelson (27, 28) in association with mouse

leukemia is also characterized by absence of immunity. This disease differs from AHA infection in its short incubation period, the absence of ascites, and the relatively high mortality in weanling mice. Pathologically, marked liver cell necrosis without perivascular cuffing is noted. Moreover, the agent unlike AHA is present in the urine readily filterable, transmissible orally; and is present in high titer.

Comparison of AHA with Lymphocytic Choriomeningitis (LCM).—The virus of lymphocytic choriomeningitis (LCM) is carried by mice, and some viscerotropic strains may produce signs in these animals which closely resemble those of infection with AHA (29, 30). The failure of AHA to cause meningitis

TABLE V

Attempts to Neutralize AHA and Neurotropic LCM with Rabbit and Guinea Pig Antisera

Method	Serum	Virus	Virus Dilution					
			0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Intracutaneous	None	LCM	—	3/3*	2/2	1/3	0/3	0/3
"	Anti-AHA rabbit	LCM	0/2	—	4/4	4/4	0/4	0/4
"	Anti-LCM guinea pig	LCM	0/2	—	0/4	0/4	0/4	0/4
Intraperitoneal	Anti-LCM guinea pig	AHA	0/2	8/8/8‡	—	—	—	—
"	None	AHA		7/7/7	—	—	—	—

* Numerator, number of mice dying in opisthotonos; denominator, number inoculated.

‡ First digit, mice with ascites; the second digit, mice with hepatosplenomegaly; the third digit, number inoculated.

in mice after intracerebral inoculation, or to infect embryonated eggs or guinea pigs, suggested that it differed from LCM. However, cross-serological and cross-immunity experiments were indicated to settle this point. The failure of anti-LCM sera to neutralize AHA infection has been mentioned above and is shown in Table V. Nevertheless attempts were made to neutralize LCM virus *in vitro* using serum from rabbits previously injected with AHA, and to neutralize AHA with anti-LCM guinea pig serum of known potency.

Ten per cent brain extracts containing LCM (Armstrong E 350-6) were diluted serially in trypticase soy broth and then mixed with equal volumes of the sera to be tested. These mixtures were allowed to stand for 1 hour at 4°C., and then mice, lightly anesthetized with ether, were inoculated intracerebrally with about 0.03 cc. of each virus-serum mixture. Those which died with opisthotonos 5 to 12 days after inoculation were considered to have succumbed to the virus.

In the intraperitoneal tests with AHA similar agent-serum mixtures were made and each mouse infected intraperitoneally with 0.2 cc. The mice were sacrificed 1 month later and examined for evidence of disease.

The results of these tests are summarized in Table V. It will be seen that the neurotropic LCM virus was completely neutralized by the anti-LCM

guinea pig serum, within the limits tested, but its titer was not decreased at all by the anti-rabbit serum. The anti-LCM guinea pig serum failed to cause neutralization or decreased severity of AHA infection by the intraperitoneal route.

Tests for protective antibody against LCM were carried out on two pools of serum obtained from patients preceding and 3 weeks following AHA inoculation. These were done by Dr. Wallace P. Rowe at the Naval Medical Research Institute (NMRI), using the technique described by him for the demonstration of protective antibody in LCM immune mice (31). The results are shown in Table VI.

TABLE VI

Attempts to Protect Mice with Mouse Sera against Viscerotrophic Strain of LCM Virus

Experiment No.	Mouse serum pool		Recipient mice showing more than 0.15 ml. fluid		Probability	
			Thoracic	Total	Thoracic	Total
1	Anti-AHA	8 wks.	5/16*	5/16	0.05‡	0.01
	Normal (O'Grady)		5/9	7/9		
2	Anti-AHA	8 wks.	5/20	7/20	0.01	0.05
	Normal (O'Grady)		11/19	12/20		
1	Anti-LCM (NMRI mice)	13 days	3/15	3/15	0.01	0.01
1	Anti-LCM (NMRI mice)	20 days	6/16	8/16	0.01	0.02
1	Normal (NMRI)		11/16	12/16		

* Thoracic plus peritoneal fluid.

‡ Numerator, number of mice showing more than 0.15 ml. fluid. Denominator, number of mice injected.

The sera were inactivated by heating at 54–56°C. for 30 minutes, and 0.1 ml. was inoculated intraperitoneally into each of a group of 9 to 20 normal 5 to 6 week old mice of the NMRI stock. 4 or 24 hours later each mouse was inoculated intraperitoneally with 0.1 ml. of a dilution of Armstrong strain LCM-infected mouse spleens, containing approximately 100 ID_{50} of virus per inoculum. On the 11th day after infection the mice were sacrificed by ether inhalation and the volume of fluid in the thoracic and peritoneal cavities was measured with a 1 ml. pipette. Mice dying before the day of sacrifice were autopsied to learn whether fluid was present. The results of the two experiments carried out by this procedure are shown in Table VI. These results indicate that, under the conditions tested, mice convalescent from AHA infection developed circulating substances which provided partial but significant protection against the viscerotropic strain of LCM virus, and that the protection observed was of a comparable order to that produced by LCM-immune mouse serum in the same experiment. For reasons stated above, AHA was thought to differ from LCM but the immunological data of the table imply antigenic similarities between the two agents or else that some of the mice employed had latent infections with LCM virus.

Further tests, using a standard complement fixation technique, were also carried out by Dr. Wallace Rowe.

A pool of sera from mice which had been infected with AHA was tested with 9 separate antigens prepared from mouse livers or spleens infected with LCM and chick embryos infected with LCM. A standard anti-LCM guinea pig serum fixed complement, in titers of 1:2 to 1:16, with all these antigens. The anti-AHA mouse serum pool likewise fixed complement with 6 of the 9 antigens to a titer of about 1:5. This pool also fixed complement with two homologous antigens prepared from the liver and spleens respectively of mice infected with AHA, but the anti-LCM guinea pig serum did not fix complement with these two heterologous antigens.

The results of these complement fixation tests further support the belief that LCM and AHA, though possibly related immunologically, are distinct.

TABLE VII

Titration of LCM in Mice Previously Infected with AHA

Time after inoculation with AHA	Stage of hepatitis	Dilution of LCM virus inoculated intracutaneously				
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
—	0	3/3	2/2	1/3	0/3	0/3
4 wks.	Acute	2/2	2/2	2/2	0/2	0/2
6–10 wks.	Convalescent	2/2	3/3	2/2	0/3	0/4

Scoring: $\frac{\text{number dying in opisthotonos}}{\text{total number of mice inoculated}}$.

Finally, tests were done to determine the susceptibility of mice, convalescent from AHA infection to challenge with LCM.

The absence of solid homologous immunity to AHA infection has been reported above. However, mice convalescent from intraperitoneal infection with LCM develop immunity to subsequent intracerebral challenge with that virus (25). The neurotropic strain of LCM was titered in three groups of mice intracerebrally, by the method previously described. One group had acute AHA infection at the time of LCM inoculation; a second group was convalescent from AHA infection (6 to 10 weeks); and a third group consisted of uninoculated mice of about the same age. The results are shown in Table VII.

It will be seen that the AHA-infected and convalescent mice were just as susceptible as controls to challenge with LCM.

The immunological and cross-immunity data just set forth support the belief that AHA and LCM are separate entities. Some of these data suggest, however, that there may be an immunological relationship between AHA and the viscerotropic strain of LCM.

RECAPITULATION AND DISCUSSION

The disease seen in mice inoculated with the agent we have called AHA has been described in the preceding paper (2). It has the characteristics of an

infection but a search for pathogenic bacteria, leptospira, protozoa, rickettsia, and viral inclusion bodies has yielded only negative findings in our laboratory. The demonstration by Dr. Anthony Morris of intranuclear and cytoplasmic inclusion bodies suggests that a protozoan may occur in mice infected with AHA. In this connection it is of interest that *Eperythrozoon coccoides* increased the virulence of the mouse hepatitis virus (MHV—S component) as described by Niven *et al.* (5), and by Nelson (32).

The failure to filter AHA and the fact that it is sedimented readily by centrifugation make it unlikely that the agent is a filterable virus. However, the possibility that even a small virus of low titer might have these characteristics, and our failure to demonstrate any known agent, required that its possible viral identity be considered. The physical and pathological findings in mice infected with AHA were similar in some respects to those in mice infected with lymphocytic choriomeningitis virus (LCM), with the mouse hepatitis virus (MHV) of Gledhill and Andrewes, and with the acute hepatitis virus of mice described by Nelson. Efforts were made to obtain positive evidence of its possible relationship to these agents. Its chief points of distinction from LCM are the longer incubation period of AHA, its failure to cause meningitis in mice inoculated by the intracerebral route, and its failure to infect guinea pigs and embryonated hens' eggs. However, strains of LCM have been encountered with differing potentials in each of these respects. For this reason the certain differentiation of AHA from LCM seemed to depend upon the demonstration of antigenic dissimilarities. It was found that both neutralizing and complement-fixing antibodies against LCM appeared in the sera of mice convalescent from AHA infection. However, anti-LCM guinea pig sera did not protect against AHA infection or fix complement with AHA antigens which were active in the homologous system. More important, mice convalescent from AHA infection manifested no resistance on subsequent infection with LCM. These differences, together with those noted previously, are considered sufficient to distinguish these two agents. The appearance of anti-LCM antibodies in mice convalescent from AHA infection suggests either some antigenic crossing between these agents or that some of the mice of the strain employed might carry LCM virus. Further experimentation will be necessary to settle these points.

AHA shares certain characteristics with MHV (3–5) and the mouse hepatitis virus described by Nelson (27, 28). It is probably identical with the hepatitis agent described by Lackey *et al.* (33). Most striking are the predilection of all these agents for the livers of mice. Occasionally there is similarity of the histopathological pictures in the livers following each infection. However, AHA usually results in more marked cellular infiltration and less necrosis than has been seen with the other agents. Moreover, homologous protecting antibodies do not develop or only in low titer following

each of the diseases. The points of difference are striking. It has been shown that mice of all strains and all ages tested are susceptible to AHA infection but with practically no mortality. In contrast MHV is reported to infect only suckling mice, or in addition weanling mice of one strain (BSVS), and in susceptible mice it is highly fatal. The mouse hepatitis virus described by Nelson produced death in 98 per cent of Princeton weanling mice but in only 4 to 12 per cent of mice of other strains. AHA infection resulted in negligible mortality in all strains and ages of mice tested and had a considerably longer incubation period than that described in the other diseases except that reported by Lackey *et al.* (33). The agents of both of these diseases have not been demonstrated to pass through a Seitz filter.

SUMMARY

A transmissible agent (AHA) causing ascites and hepatitis in mice has been described. No known pathogenic bacteria, fungi, protozoa, leptospira, rickettsia, or viruses have been demonstrated in the infected mice. AHA does not pass through a Seitz filter and differs in most respects from the agents previously described which produce hepatitis in mice.

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