STUDIES ON THE AGENT OF INFECTIOUS HEPATITIS

I. Propagation of the Agent in Tissue Culture and in the Embryonated Hen's Egg*

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Investigations into the etiology of infectious hepatitis conducted during World War II, leave little doubt that the disease is caused by a virus.

The agent capable of inducing infectious hepatitis in human volunteers was found to be present in the duodenal contents (1), in the blood (2), and in the stools of patients during the acute stage of the disease (3–6). The agent could be transmitted in series from volunteer to volunteer (7) and materials taken from patients retained their infectivity after passage through bacteria-tight filters (7–9). In spite of all efforts, the agent has not been established unequivocally in experimental hosts (10, 11). The recorded data as well as many unpublished attempts at isolation have been collected recently (12). It is apparent that most of the common laboratory animals as well as many more unusual species have been tested. The infectious materials were administered by all the usual routes. In some studies the animals were conditioned by certain diets or drugs known to induce some liver damage. Although most of these efforts have failed, a few results were reported suggestive of successful transmission of an agent to hogs (13), guinea pigs (14), chick embryos (15–17), canaries (18, 19), and rats maintained on a deficient diet (20). In none of these has the nature of the isolated agent been definitely identified by neutralization tests with acute and convalescent sera of infectious hepatitis patients, or by induction of hepatitis with the isolated agent in human volunteers, with the exception of possibly one study (21). None of the reports has been confirmed by independent investigators (12).

The first attempts to propagate infectious hepatitis virus in chick embryo or rabbit liver tissue culture in this laboratory date back to March, 1945. The studies have been carried out intermittently since that time, the interruptions being caused mainly by the unavailability, at certain stages of the work, of volunteers, who were needed in order to ascertain the presence of virus in the

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preparations. It will be shown in this report that an agent inducing mild changes in hepatic function in volunteers has been carried in series through cultures of minced chick embryos and roller-tube cultures of mammalian liver cells, and that this agent could subsequently be transferred to the amniotic cavity of the chick embryo. The present communication deals with the cultural aspects of the studies, and the next paper of this series will discuss the clinical features of the disease induced by the tissue culture agent in volunteers (22).

Methods and Materials

Virus.—Sera or suspensions of stools obtained from patients in the acute stage of infectious hepatitis served as the source of virus. The Akiba strain was obtained from an outbreak of the disease in a summer camp (8) and the NL strain from a similar outbreak at an institution for mentally retarded males (23). The serum was collected aseptically. Stool extracts were centrifuged at 4000 R.P.M. for 30 minutes and filtered through a Seitz EK pad. That the starting materials contained virus was proven by infection of volunteers.

Minced Chick Embryo Tissue Culture.—The medium used in this type of tissue culture was that described by Simms and Sanders (24) for the cultivation of miscellaneous viruses. Shortly after these studies were initiated the ox-serum ultrafiltrate, combined with the complete set of Simms salt solutions (UF/3×7) became available commercially. This preparation was used throughout the remainder of this work.[1] Chick embryos at the 10th to the 13th day of incubation were finely minced with strabismus scissors after removal of the eyes, beak, and claws. The minced tissue was washed 3 times in UF/3×7 in graduated centrifuge tubes. After the last washing the tissue fragments were allowed to settle, and the supernatant fluid was largely removed; the amount remaining being equal to twice the volume of the minced tissue. Six-tenths of a milliliter of this 33 per cent tissue suspension was then added to 20 ml. of UF/3×7 in 50 ml. Erlenmeyer flasks, thereby reaching a ratio of tissue to fluid of approximately 1 to 100. The flasks were closed with rubber stoppers and incubated at 37°C. for 48 hours before inoculation in order to eliminate bacterially contaminated cultures. The infectious materials were then introduced into the flasks in 1 ml. amounts. The infected cultures were incubated at 37°C. for 1 week and then removed to a cold room at 4°C. until transfers were made. Of this material, 1.2 ml. of fluid and tissue fragments, taken at random, were used for further passage. Subsequently the cultures were stored at −20°C. for preservation.

In the earlier experiments small bits of tissue were frequently transplanted into roller tubes in order to obtain evidence of viability of at least some of the tissue elements. Halos of outgrowing cells were obtained in the majority of transplants from all cultures as late as 2 to 3 weeks after preparation of the minced chick embryo culture. Because of the regularity of these results viability tests were omitted from most of the later experiments.

Tissue Cultures of Rabbit Liver Cell.—These were set up according to the technic developed and described by two of us (25). The livers of 1- to 4-day-old rabbits were removed aseptically and minced. The pieces, of an average diameter of 1 mm., were washed in balanced salt solution and introduced into test tubes, 18 mm. × 150 mm. About 40 such pieces were scattered about the wall of the lowest third of the tube, which had been moistened with heparinized rabbit plasma. On addition of chick embryo extract and rotation of the tube a clot formed in the plasma, which held the bits of liver to the wall of the tube. Nutrient fluid was now added in a quantity of 1.6 ml. per tube, of which 40 per cent was normal rabbit serum and 60 per cent balanced salt solution, essentially identical with Gey's solution except for the addi-

[1] Microbiological Associates, Inc., Flemington, New Jersey.

tion of aspartic acid to suppress the growth of fibroblasts (26). In such cultures columns of epithelial cells appeared at the margins of the explants, after a few days. These grew and coalesced into sheets of cells, attaining a maximum radius of about 4 mm. in about 10 days.

Into these cultures the infectious agent was introduced by the addition of 0.2 ml. of serum containing the Akiba strain of virus. The cultures were fed after 3 to 4 days of rotation in the roller apparatus by withdrawing the nutrient fluid and replacing it with fresh rabbit serum–salt solution mixture. After further incubation for a total of 1 week, the nutrient fluid was collected to serve as passage material for new liver cell cultures. The fluid was diluted 1:5 in fresh nutrient fluid and 0.2 ml. was used for further inoculation. The nutrient fluids removed at the end of the incubation periods were stored at −20°C. for preservation.

Amniotic Inoculation of Chick Embryos.—The 10th passage chick embryo tissue culture infected with Akiba virus was transferred to the amniotic cavity of the developing chick embryo, using 0.2 ml. inocula, and passed several times in this medium. Embryos at the 7th to 8th day of incubation were employed. The technic of inoculation corresponded to that described for infection with mumps virus (27). After further incubation of the eggs at 36 to 37°C. for 1 week, the whole contents of the eggs, except for the albumen, were harvested and emulsified in a Waring blendor with 40 ml. of buffered saline. Two-tenths ml. of this emulsion was passed to new groups of eggs. The 3rd passage material was tested in human volunteers. In some of the later passages only the amniotic fluids were collected and passed (28). A control series of passages was initiated by amniotic injection of saline solution instead of the 10th passage tissue culture material. In all other respects these transfers were handled like the infectious hepatitis series.

Assay of Infectivity in Human Volunteers.—This phase of the work is described in detail in the second paper of this series (22).

EXPERIMENTAL

Experiments with the Akiba Strain.—

Three series of tissue cultures were initiated in March, 1945. Two of these consisted of minced chick embryo in Simms-Sanders medium, one being infected with Akiba stool filtrate and the other with Akiba acute stage serum, both of known infectivity for volunteers. The third series was a newborn rabbit liver cell culture in roller tubes, seeded with Akiba serum. The stool extract had proved too toxic for the epithelial cells. After the first 6 passages, at weekly intervals, the chick embryo tissue fragments were homogenized in their media by exposure to intense sonic vibration for 3 minutes.[2] These emulsions, as well as the 6th passage nutrient fluids of the liver cultures, were tested for bacterial sterility, both aerobically and anaerobically, and for safety by intraperitoneal, intracerebral, and subcutaneous injections of mice and guinea pigs. They represented a dilution of the original inoculum of acute phase serum or stool filtrate of about $10^{-7.7}$. The materials were then stored at −20°C. until volunteers became available 20 to 27 months later. The following three experiments were then performed with these materials.

Five volunteers were given a pool of equal parts of all three tissue culture series, 6 ml. orally, diluted in chocolate milk, and 1 ml. subcutaneously. Three of these individuals, as shown in Table I, showed mild signs of illness, beginning on the 21st to 25th day after inoculation and lasting for from 4 to 19 days.

[2] The oscillator employed was manufactured by the Raytheon Company of Boston, Massachusetts.

All five showed intermittent mild bilirubinuria and urobilinogenuria beginning on the 14th to 20th day and lasting for from 18 to 32 days. No abnormalities were found in the serum tests for hepatitis.

As a result of the suggestive findings in the first experiment the individual tissue culture preparations were tested separately. Groups of three volunteers each received 4 ml. orally and 1 ml. subcutaneously of one of the tissue culture lines. The results are recorded in Table II. It can be seen that mild clinical signs and positive laboratory findings were observed in one or more of the volunteers of each group, the time relationships being similar to those encountered in the first experiment. Aside from bilirubinuria and urobilinogenuria cephalin-

TABLE I

Results of Inoculation of Pooled Tissue Culture Materials 6th Passage

Subject	Clinical signs	Laboratory findings	Remarks
	days after inoculation	*days after inoculation*	
1	25 to 44	17 to 39	Slight liver tenderness. Bilirubinuria. Urobilinogenuria
2	21 to 40	20 to 46	" "
3	22 to 26	18 to 36	Malaise, anorexia. Bilirubinuria. Urobilinogenuria
4	—	18 to 50	Bilirubinuria. Urobilinogenuria
5	—	14 to 44	" "

cholesterol and thymol flocculation tests were positive in some of the cases and suggestive elevations of serum bilirubin and retention of bromsulfalein were recorded.

A third group of volunteers were infected with a 100-fold dilution of a pool of equal parts of the two lines of chick embryo tissue culture, 4 ml. orally and 1 ml. subcutaneously. This increased the dilution factor of the original seed material to $10^{-9.7}$. Two of these volunteers showed abnormal liver function tests between the 32nd and 60th day after administration, but none developed clinical signs of illness (Table III). Both cases revealed bromsulfalein retention of 10 and 15 per cent respectively; one showed significantly increased serum bilirubin and the other positive serum flocculation tests.

These tests seemed to indicate that all three lines of tissue cultures contained an agent which produced mild signs of hepatitis in 14 out of 18 individuals, either on clinical or laboratory evidence or both. The incubation periods of 21 to 30 days resembled those encountered in infectious hepatitis under epidemic

conditions. However, at this early stage in the passage series it could not be stated with assurance whether this agent had actually multiplied in the media

TABLE II

Results of Inoculation of Individual Tissue Culture Materials (Akiba Strain)
6th Passage

Culture	Subject	Clinical signs	Laboratory findings	Remarks
		days after inoculation	*days after inoculation*	
Chick embryo Akiba stool	6	23 to 36	20 to 62	Liver tenderness. Bilirubinuria. Positive flocculation tests. Elevated serum bilirubin.
	7	21 to 30	14 to 48	" " Bsf. *±
	8	—	18 to 62	Bilirubinuria. Positive flocculation tests
Chick embryo Akiba serum	9	—	—	—
	10	30 to 36	19 to 48	Liver tenderness. Bilirubinuria. Positive flocculation tests Bsf. ±
	11	—	16 to 42	Bilirubinuria. Bsf. 10 per cent
Rabbit liver Akiba serum	12	—	34 to 62	Positive flocculation tests
	13	—	—	—
	14	25 to 32	—	Slight liver tenderness

* Bromsulfalein retention.

TABLE III

Results of Inoculation of Chick Embryo Tissue Culture (Akiba Strain)
6th Passage
Diluted 1:100

Subject	Clinical signs	Laboratory findings	Remarks
	days after inoculation	*days after inoculation*	
15	—	—	—
16	—	—	—
17	—	32 to 40	Bilirubinuria. Bsf. 10 to 12 per cent. Elevated serum bilirubin
18	—	46 to 60	Bilirubinuria. Bsf. 15 per cent. Positive flocculation tests

or whether it had been carried over from the original seed material. Further passages were, therefore, indicated. Since the two chick embryo tissue culture series seemed to yield comparable results, and since these, in turn, resembled

those obtained with the liver cultures, the former two were combined and carried thus in further transfers, and the latter line was discontinued as it was more cumbersome. These experiments were hampered once again by the lack of volunteers. When new volunteers became finally available new sublines were initiated from the 6th passage material which had been stored in the interim at −20°C. for 18 months. Four volunteers were each given by mouth 4 ml. of 8th passage chick embryo tissue culture, and another four, a similar amount of the 10th passage material. In both instances the tissue fragments had been emulsified in the liquid medium by intense sonic vibration for 3 minutes. No subcutaneous injection was given in addition to the oral administration in this

TABLE IV

Results of Inoculation of Chick Embryo Tissue Culture (Akiba Strain) 8th and 10th Passages

Culture	Subject No.	Clinical signs	Laboratory findings	Remarks
		days after inoculation	*days after inoculation*	
8th passage	19	33 to 61	34 to 46	Enlarged, tender liver. Positive flocculation tests
	20	19 to 54	26 to 54	Enlarged, tender liver. Positive flocculation tests
	21	12 to 44	27 to 38	Enlarged, tender liver. Positive flocculation tests
	22	—	—	—
10th passage	23	26 to 57	30 to 41	Enlarged, tender liver. Positive flocculation tests
	24	36 to 61	—	Enlarged, tender liver
	25	—	—	—
	26	—	—	—

and the subsequent experiments. These preparations represented dilutions of the original seed material of about $10^{-10.4}$ and 10^{-13}, respectively. The results are shown in Table IV. Three and two of the volunteers in the respective groups developed enlarged, tender livers and positive serum flocculation tests within 12 to 36 days.

This test confirmed the earlier findings and it seemed quite definite now that an agent had been propagated in the chick embryo tissue culture. It was felt, therefore, that it might grow in the intact embryo as well. Consequently, 10th passage tissue culture was injected in 0.2 ml. amounts into the amniotic cavity of 8-day-old chick embryos and passed three times as described in the section on methods. This raised the dilution factor of the original seed material to approximately 10^{-20}. Five volunteers each received orally 4 ml. of a suspension of the whole contents of the egg after the 3rd amniotic passage. Three of these showed signs of hepatitis without jaundice, with an average incubation period

of 20 days as seen in Table V. This line is being continued with various materials from the chick embryo being passed separately.

Experiments with the NL Strain.—Another line of chick embryo tissue culture was initiated more recently. It was seeded with serum taken from patients during the acute stage of infectious hepatitis during an outbreak of the disease

TABLE V

Results of Inoculation of Amniotic Passage Material (Akiba Strain) 10 Tissue Culture and 3 Amniotic Passages

Subject	Clinical signs	Laboratory findings	Remarks
	days after inoculation	*days after inoculation*	
27	9 to 49	19 to 55	Enlarged, tender liver. Positive flocculation tests
28	19 to 49	28 to 61	Enlarged, tender liver. Positive flocculation tests
29	32 to 56	28 to 55	Enlarged, tender liver. Positive flocculation tests
30	17 to 44	—	Enlarged, tender liver
31	31 to 44	—	Enlarged, tender liver

TABLE VI

Results of Inoculation of Chick Embryo Tissue Culture (NL Strain) 10th Passage

Subject	Clinical signs	Laboratory findings	Remarks
	days after inoculation	*days after inoculation*	
32	25 to 49	24 to 49	Enlarged, tender liver. Positive flocculation tests
33	9 to 49	32 to 74	Enlarged, tender liver. Positive flocculation tests
34	38 to 47	32 to 55	Intermittently enlarged liver. Positive flocculation tests
35	—	—	—
36	—	—	—

at Institution NL. The serum had proved infectious for volunteers. The virus was carried through ten consecutive passages at weekly intervals in the manner described. The homogenized culture of the last transfer was given to five volunteers orally in 4 ml. amounts. Three of these developed mild signs of clinical hepatitis without jaundice, two with supporting laboratory evidence. An average incubation period of 27 days was recorded. The data are summarized in Table VI.

Attempts to Identify the Agent.—Various attempts have been made to identify

the agent which had been propagated in the tissue and egg cultures. These can be divided into two groups: (1) determination of resistance of volunteers convalescent from the "tissue culture disease" to reexposure to infectious hepatitis virus as obtained in acute stage sera of human cases of hepatitis with jaundice (NL strain); and (2) search for specific diagnostic tests.

The first approach could not be used to any large extent. Only a total of seven volunteers, who had been exposed to culture virus in the past, agreed to submit to a second exposure to natural infectious hepatitis virus. These were challenged in two groups of three and four, respectively, 2 months apart. At each date control subjects with negative histories of infectious hepatitis were likewise exposed, four in the first experiment, and two, with negative skin tests (28), in the second. The results of the exposures are combined in Table VII.

TABLE VII

Resistance to Natural Virus Following Recovery from Infection with the Cultivated Agent

First exposure to cultivated virus			Second exposure to natural virus (NL strain)			
			Hepatitis			
Strain	Result	No. of volunteers	With jaundice	Without jaundice		No hepatitis
				Positive laboratory findings	Negative laboratory findings	
Akiba	Hepatitis	4	0	0	0	4
Akiba	No hepatitis	1	1	0	0	0
NL	Hepatitis	2	0	0	0	2
None	—	6	2	3	1	0

Of the indviduals volunteering for a second exposure, six had shown clinical and laboratory signs of illness in the first test and these remained entirely well on second challenge with natural virus 6 to 8 months later. On the other hand, the seventh volunteer, who failed to reveal signs of illness after exposure to culture virus, became jaundiced as a result of the oral administration of the natural infectious hepatitis virus. Of the six control subjects two developed jaundice, three hepatitis without jaundice confirmed by laboratory tests, and one revealed very mild clinical signs of infection without laboratory support. These data are summarized in Table VII.

The attempts to develop specific serological tests with the culture materials on hand included, with no success as yet, a search for complement fixation antigens and hemagglutinating properties of the agent. All tests were negative, although in some experiments the tissue culture materials had been concentrated 80- to 100-fold by pervaporation with intermittent dialysis or by sedi-

mentation in the high speed centrifuge at 30,000 R.P.M. for 1 hour. Efforts to detect virus by certain activities which could be employed for neutralization tests with acute stage and convalescent infectious hepatitis sera, likewise, have failed thus far. The agent had no apparent effect upon the outgrowth of cells from transplants of the tissue culture fragments to roller tubes. It is realized, of course, that these fragments contained many different types of cells and that the virus may have had a destructive effect only on some. Although there was some evidence of liver damage in some of the chick embryos of the infectious hepatitis series (Akiba) by the 3rd amniotic passage, but not in chick embryos injected with similar, but non-infected passage materials, these lesions are too inconsistent to be considered for neutralization tests.

Most encouraging results were obtained recently, however, with skin test antigens derived from 5th to 7th passage amniotic fluids collected from chick embryos infected amniotically with the Akiba strain. A preliminary report of these findings has been published elsewhere (28). These experiments revealed a positive skin test in all convalescents of infectious hepatitis tested thus far regardless of whether they developed the disease spontaneously or after experimental exposure to the Akiba and NL strains as contained in human serum and stool extracts, or in tissue culture.

DISCUSSION

The tests recorded indicate that an agent which is capable of inducing mild hepatitis in volunteers has been propagated in chick embryo and rabbit liver tissue culture and in the amniotic cavity of the chick embryo. The disease produced is discussed in greater detail in the second paper of this series (22). Whether or not this agent is identical with that causing infectious hepatitis cannot be stated with absolute certainty at the present time. However, there are several points which strongly suggest that this is the case: (*a*) The incubation periods obtained with the tissue culture agent are similar to those encountered in the natural disease; *i.e.*, 9 to 37 days. (*b*) Although none of the "tissue culture cases" developed jaundice, hepatitis following transmission of infectious hepatitis virus as contained in stools and serum during the acute stage of the natural disease is by no means regularly accompanied by jaundice. Hepatitis without jaundice is not uncommon under epidemic as well as under experimental conditions. One may consider that the complete absence of jaundice in the tissue culture cases denotes, possibly, some attenuation of the virus. (*c*) Similar results were obtained with two strains of virus obtained in two different epidemics. (*d*) Re-exposure of six convalescents of the culture virus disease to fully virulent natural virus failed to induce hepatitis, whereas another volunteer, exposed to tissue culture virus but failing to show any signs of illness, and five out of six controls developed clinical and laboratory signs of disease. The number of volunteers challenged is small, but the results are highly

suggestive of the identity of the tissue culture virus with the infectious hepatitis agent. (*e*) Finally this suggestion has been supported further recently when it was found (28) that amniotic fluids of the 5th to 7th amniotic passage series, after inactivation by ultraviolet light, yielded a skin test antigen which appeared specific in that positive results were obtained in convalescents of spontaneous as well as experimental infectious hepatitis, regardless of whether natural or tissue culture virus was used for infection. Convalescents of homologous serum hepatitis responded to this antigen with an incidence not exceeding that found in a population chosen at random.

Thus it is felt that the evidence available at present supports strongly the suggestion that the virus propagated in tissue culture and in the chick embryo is identical with infectious hepatitis virus. Final identification, however, must await the development of specific serological tests. All attempts to develop such tests have failed thus far, most likely because of insufficient quantities of virus present in the preparations studied.

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SUMMARY

Two viral agents have been procured from patients with infectious hepatitis in two widely separated outbreaks of the disease by transfer of acute stage serum and stool filtrates to and passage in tissue cultures of rabbit liver cells in roller tubes and minced chick embryos in Simms-Sanders medium followed by passage in the amniotic cavity of the chick. Cultures of both agents, designated the Akiba and NL strains of virus, induced mild hepatitis without jaundice in the majority of volunteers tested after an incubation period of from 9 to 38 days. Although these agents have not been identified definitely as the virus of infectious hepatitis, the available evidence, as discussed, is compatible with the suggestion that such they are.

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