THE PROPAGATION OF THE VIRUS OF EPIZOOTIC HEMORRHAGIC DISEASE OF DEER IN NEWBORN MICE AND HeLa CELLS*, ‡

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In an earlier paper (1), two of us (R. E. S. and L. G. M.) reported failure to transmit the virus of epizootic hemorrhagic disease of deer (EHD) to suckling mice or to propagate it in tissue cultures of deer kidney cells. It is apparent from work now to be reported that insufficient attention was paid in the earlier experiments to relatively minor signs of illness in suckling mice inoculated with virus directly of deer origin and that perhaps the wrong tissue culture cell system was used in attempting propagation of the virus. The present paper reports the successful infection of suckling mice with EHD virus and propagation of the virus in HeLa cell culture.

Materials and Methods

Virus.—Deer 1-39, typically ill of the New Jersey strain of EHD after a 6 day incubation period, was sacrificed on the 2nd day of illness. The spleen of this animal, suspended to 10 per cent in 0.75 per cent bovine albumin buffered to pH 7.2, was centrifuged at 2000 RPM for 10 minutes and the supernatant constituted the starting virus for initiating infections in both the newborn mice and the HeLa cells.

Mice.—Swiss mice inoculated on the same day they were born were infected intracerebrally with 0.02 cc amounts of a 10^{-2} dilution of the original deer spleen or, in later serial passages, with 10^{-2} dilutions of the brains of mice of the preceding passage.

Deer.—The experimental deer were obtained in nature as fawns and were bottle-fed on cows' milk until weaning time. After this they were maintained on a diet of hay and a grain mixture known as omolene (Ralston Purina Co., St. Louis). They were between 8 and 10 months old when used, and were maintained in pens, 7 feet high, 6 feet wide, and 9 feet deep, that were lined with 14 gauge welded wire of 2 by 1 inch mesh.

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HeLa Cell Cultures.—The HeLa cell line of The Rockefeller Foundation Virus Laboratories was employed. This line had been used successfully for the propagation of the majority of the arbor viruses (2). Fifty thousand cells per ml were seeded in culture fluid comprised of 97 per cent Eagle's medium and 3 per cent fetal bovine serum and inoculation with EHD virus was made simultaneously with the seeding of the culture tubes. The inoculum consisted of increasing dilutions of infected deer spleen suspended in 0.75 per cent bovine albumin buffered to pH 7.2. The dilutions were from 10^{-1} to 10^{-7} and three tubes were used for each dilution. The maintenance fluid was changed daily and cultures were observed for 3 weeks. The time of serial transfer of cultures was determined by the time of appearance of a cytopathic effect.

Complement Fixation System.—The complement-fixing antigen was made from the brains of mice ill from EHD virus infection using sucrose-acetone extraction which is currently in use for work with arbor viruses (3). These antigens were satisfactory for complement fixation but did not contain hemagglutinins for goose red blood cells when tested at various pHs, including following treatment with protamine sulfate.

Immune serum was obtained either from deer that had recovered from infection with the New Jersey or South Dakota strain of EHD or from adult mice immunized with live New Jersey strain EHD virus. The immunization of mice was effected by inoculating them three times intraperitoneally at intervals of 30 and 21 days with 0.3 cc of the supernatant fluid of 10 per cent suspensions of EHD virus-infected suckling mouse brains in saline. Serum was obtained from the mice 7 days after their third injection.

The complement fixation tests were performed by a semimicro technique in plastic plates with depressions using increasing twofold dilutions of sera and antigens in box or checkerboard titrations. The first dilution of the sera or antigens was usually 1:4. Into individual depressions on the plates were placed one drop of the serum, two full units of complement in two drops, and one drop of the antigen dilution. The deer sera were previously inactivated at 56°C for 30 minutes and the mouse sera at 60°C for 20 minutes. After incubating at 4°C overnight, the hemolytic system consisting of two drops of sensitized sheep erythrocytes was added while the plates were shaken on an automatic shaker. The plates were incubated at 37°C for 30 minutes and were then placed at 4°C until read.

Virus Neutralization Tests.—The virus neutralization tests were conducted in HeLa cell cultures. The sera, previously inactivated as outlined in the preceding section, were used undiluted and were added to increasing tenfold dilutions of tissue culture EHD virus. The serum-virus mixtures were incubated at 37°C for 1 hour before being added to the tissue culture system which consisted of 50,000 HeLa cells per ml seeded in culture fluid comprised of 97 per cent Eagle's medium and 3 per cent fetal bovine serum. The cultures were observed for 3 weeks, during which time the maintenance fluid was changed daily.

RESULTS

Transmission of EHD Virus in Suckling Mice.—Four litters, comprised of a total of 32 newborn Swiss mice, were inoculated intracerebrally with a suspension of spleen from deer 1-39, ill of the New Jersey strain of EHD. On the 6th day, two mice which exhibited mild tonic and clonic convulsions were sacrificed and their brains, homogenized in buffered 0.75 per cent bovine albumin, were administered intracerebrally to 48 newborn mice. On the 7th day, 16 more mice of the initial passage, which were by now exhibiting tremors and irregular respiration, were sacrificed. The brains of these mice were stored frozen, pending the results of the passage of the previous day. Of the remaining

14 mice of the original passage, 8 died between the 11th and 15th days and the other 6 remained healthy during 21 days of observation.

The mice of the second passage, those inoculated with brain suspension from the first two mice to sicken in the first passage, became ill on the 4th and 5th days following inoculation. Twenty-eight of these died on the 5th day and the remaining 20 moribund mice were sacrificed on the same day. Their brains were saved for virus stock to be used for further passages and for the preparation of complement-fixing antigen.

TABLE I

Serial Passage of EHD Virus in Suckling Mice (0 to 1 day old)

Passage	No. of mice inoculated	Result		
		Incubation period	No. sacrificed when moribund	No. dying
		days		
1	32*	6–7	18	8/14‡
2	48§	4	20	28/28
3	177	3–4	92	85/85
4	137	3	127	10/10
5	48	3–4	35	13/13

* 0.02 cc 10^{-2} dilution spleen deer 1-39 intracerebrally.

‡ $\frac{\text{Mice dead}}{\text{Mice inoculated}}$

§ 0.02 cc 10^{-2} dilution brains from preceding mouse passage intracerebrally.

Mice of the third and subsequent serial passages have sickened regularly on the 3rd or 4th day after inoculation and those not sacrificed for virus have succumbed. A record of the results of the first five mouse passages is given in Table I.

Mice infected with EHD virus have shown an illness characterized by loss of postural reflexes, irregular respiration, cyanosis, and tonic and clonic convulsions. All save the few surviving in the first passage have remained sick until death on from the 1st to the 5th day after the initial appearance of signs of illness. Since, with higher dilutions of virus, some mice occasionally lived for as long as 15 days postinoculation, we have kept our mice under observation for 21 days before counting them as negative to infection.

Serological Evidence That the Agent Infective for Suckling Mice Is the EHD Virus.—In order to establish a relationship between the mouse infective agent from deer spleen and the EHD virus, complement-fixing antigen was prepared, as described earlier, from the brains of moribund mice. This antigen was tested

for its capacity to fix complement with sera from deer that had recovered from EHD as well as with sera of mice immunized to the mouse-passaged agent. Appropriate controls consisting of serum from a normal deer, mouse brain antigen prepared from an unrelated arbor virus, and antiserum to the South Dakota strain of EHD virus were included in the tests. The results are shown in Table II.

TABLE II

Fixation of Complement by Sera of Deer Recovered from EHD When Tested with Infected Mouse Brain Complement-Fixing Antigen

Serum			Complement-fixing antigen	
Deer No.	Immune to	Month and year of sample	Brains of mice infected with New Jersey strain EHD virus	Brains of mice infected with unrelated virus
2-7	New Jersey strain EHD virus	Mar., 1956	8/32*	0
7-4	New Jersey strain EHD virus	Feb., 1959	16/32	0
8-1	New Jersey and South Dakota strains EHD virus	Dec., 1958	8/32	0
8-6	South Dakota strain EHD virus	Feb., 1958	16/32	0
1-05	New Jersey and South Dakota strains EHD virus	Feb., 1959	8/32	0
1-38	New Jersey strain EHD virus	Jan., 1962	64/32	0
1-34	Normal	July, 1960	0	0
Pooled sera of mice immunized to New Jersey strain EHD virus			128/16	0

* $\frac{\text{Reciprocal of serum titer}}{\text{Reciprocal of antigen titer}}$

As shown in Table II, the sera from deer recovered from infection with EHD, either the New Jersey or South Dakota strain, fixed complement in tests with antigen prepared from the brains of mice ill with an agent derived from the spleen of an EHD virus-infected deer. The serum from a normal deer failed to react with the antigen, nor did any of the sera react with a mouse brain antigen prepared from an unrelated virus. It is of interest, so far as concerns the possibility that the agent transmitted to mice might have been a latent virus other than EHD, that the reacting convalescent deer sera were obtained in 4 separate years spanning a period from 1956 to 1962. The complement-fixing antigen failed to differentiate between antibodies against the New Jersey and South Dakota strains of EHD virus. The serological findings just outlined, considered with a test of the infectivity of the mouse-passaged agent for deer, to be described next, indicate that the mouse pathogenic agent under discussion is indeed the EHD virus.

Direct Test of Mouse Pathogenic Agent for Infectivity in Deer.—Five cc of a 10 per cent suspension of infected mouse brain of the fourth serial mouse passage was given intramuscularly to deer 1-41. The brains had been harvested from moribund mice on the 3rd day after inoculation and were suspended in 0.75 per cent bovine albumin buffered to pH 7.2. The titer of this brain suspension in HeLa cell culture was greater than 10^{-8} $TCID_{50}$ per ml. The inoculated deer developed no definitely characteristic signs of illness during a period of observation of 57 days. At the end of this time, it was bled and its serum gave a 16/32 positive complement fixation test with mouse brain antigens prepared from both the mouse passage and HeLa cell passage culture lines of New Jersey strain EHD virus. The serum had no antibodies for control normal mouse brain. Unfortunately we had not obtained a preinoculation blood sample from this particular deer. However, the animal had been reared in captivity, there has been no recognized EHD in New Jersey since the outbreak in 1955, and we have never previously encountered EHD antibodies in a deer without known illness. These considerations make it appear extremely unlikely that the antibodies present in the serum of deer 1-41 arose from any experience with EHD virus antedating his exposure to the mouse-passaged agent. Rather it seems likely that the deer underwent a subclinical and asymptomatic infection by the mouse-passaged EHD virus. Whether the mildness of this infection resulted from attentuation of the virus during the four serial mouse brain passages to which it had been subjected will have to be determined later this year when our new crop of deer become available.

The Propagation of EHD Virus in HeLa Cells.—HeLa cell cultures were inoculated with tenfold dilutions, from 10^{-1} to 10^{-7}, of the same suspension of infected deer spleen used to initiate the suckling mouse passages, using three tubes for each dilution. A cytopathic effect appeared in 4 days in the lower dilution culture tubes of the first passage and in 8 days in the highest positive dilutions. The cells were observed for 21 days but the maximum cytopathic effect was noted at about the 14th day. Based on the occurrence of a cytopathic effect, the initiating 10 per cent deer spleen suspension contained $10^{4.5}$ $TCID_{50}$ of cytopathic agent per 0.1 ml. The cytopathic effect first became evident as granulations in cells in scattered areas of the cell sheet. These rounded up, contracted, and finally separated from the walls of the tube forming holes in the cell sheet.

The second serial passage in HeLa cell cultures was prepared, using infected fluid taken on the 7th day from the 10^{-2} dilution tubes of the first passage using increasing dilutions to 10^{-6}. The third serial passage made by infecting cell cultures with fluid from the second passage was used to infect an experimental deer, as will be described later. The virus passage line established in HeLa cell cultures as just outlined has been passaged regularly and is as easily maintained as that in suckling mice. The cytopathic effect in later passages appears 1 to 4 days earlier than it did in the first passage cultures.

The agent was also grown in a HeLa cell monolayer system in which 200,000 cells per ml were seeded in a medium composed of 30 per cent pooled human adult serum, 10 per cent fetal bovine serum, 15 per cent tryptose phosphate broth, and 45 per cent Hanks's balanced salt solution with added penicillin, streptomycin, and mycostatin. After 48 hours' incubation, this medium was replaced by fluid composed of 97 per cent Eagle's medium and 3 per cent fetal bovine serum before inoculating with the EHD agent. In such a monolayer system, a cytopathic effect could be observed usually within 48 hours of inoculation.

TABLE III

Neutralization by the Sera of EHD-Recovered Deer of the Agent Propagated in HeLa Cell Culture

Test No.	Serum		Virus (3rd HeLa cell passage)	
	Deer No.	Immune to	Titer of virus	Log neutralization index
1	2-7	New Jersey strain EHD virus	$<10^0$	>6
	8-1	New Jersey and South Dakota strains EHD virus	$<10^0$	>6
	8-6	South Dakota strain EHD virus	$10^{-3.5}$	2.5
	1-05	New Jersey and South Dakota strains EHD virus	$10^{-0.5}$	5.5
	1-38	New Jersey strain EHD virus	$<10^0$	>6
	1-34	Normal	10^{-5}	1
		None, diluent	10^{-6}	
2	7-4	New Jersey strain EHD virus	$10^{-1.5}$	4
	1-34	Normal	$10^{-4.5}$	1
		None, diluent	$10^{-5.5}$	

Serological Evidence That the Agent Propagated in HeLa Cell Culture Is the EHD Virus.—In order to establish a relationship between the HeLa cell infective agent from deer spleen and the EHD virus, attempts to neutralize the agent in HeLa cell culture with sera from deer recovered from EHD were made. The technique of the neutralization test has been described earlier. The sera used were from deer recovered from either the New Jersey or South Dakota strain of EHD alone, or from animals that had been sequentially infected with both strains of the EHD virus. Serum from a normal deer was included as a control. The results obtained are outlined in Table III.

As shown in Table III, the sera of all deer that had recovered from infection with the New Jersey strain of EHD neutralized the agent propagated in HeLa cells. The sera of a deer recovered from infection with the South Dakota strain

of EHD neutralized the HeLa cell agent to some extent. The serological data, so far as they went, indicated that the agent propagated in the HeLa cell cultures was indeed the New Jersey strain of EHD virus. The finding that serum from a deer recovered from South Dakota strain EHD neutralized the New Jersey strain of virus to some extent had not been apparent in earlier cross-neutralization tests in deer (1), probably because these were not conducted in the quantitative manner that the HeLa cell tests were.

Direct Test of HeLa Cell Pathogenic Agent in Deer.—Four cc of infected HeLa cell culture fluid containing suspended cells was administered intramuscularly to deer 1-40. This was third passage level material and contained $10^{5.7}$ $TCID_{50}$ per 0.1 ml. The inoculated deer became typically ill of EHD on the 8th day and died on the 17th day postinoculation. The incubation period in this animal was thus similar to that observed in our earlier work with EHD virus directly of deer origin and the period of illness undergone before death finally ensued was within the range seen in our earlier subacute cases (1). During its illness, the animal exhibited the signs characteristic of EHD caused by virus of deer origin: anorexia, roughening of the coat, marked injection of the conjunctiva and mucosa of the lips, salivation, and marked prostration.

The findings encountered at autopsy in deer 1-40 were like those seen in our earlier work. There were moderate petechial hemorrhages in the subcutaneous tissues and in the tendon sheaths at the joints. There was tarry blood in the gastrointestinal tract, excess fluid in the pericardial sac, with petechiation of the heart muscle, some hemorrhages in the diaphragm and parietal pleurae, and intense congestion of the tracheal mucosa. There were submucosal hemorrhages involving the ventral aspect of the tongue and marked petechiation of the cartilages of the ears. In the gross the brain appeared normal.

It appeared from both the signs of illness and the autopsy pictures that deer 1-40 underwent typical EHD as a result of infection with the HeLa cell pathogenic agent and that the agent propagated in HeLa cells was indeed the EHD virus. Various organs and tissues of deer 1-40 were saved at autopsy and attempts were made to reisolate virus from them. The results of these tests will be outlined in the following section.

Reisolation of Virus from a Deer Infected with EHD Virus Grown in HeLa Cells.—Blood was drawn from deer 1-40 on the 7th day of its illness, at a time when death appeared to be imminent, and gluteal muscle, lung, heart, brain, kidney, liver, and spleen were obtained at autopsy shortly following the animal's death on its 9th day of illness. Each organ obtained at autopsy, as well as the blood clot obtained 2 days before death, was suspended separately in 0.75 per cent bovine albumin buffered at pH 7.2. Dilutions of these suspensions, as well as the antemortem blood serum, from 10^{-1} to 10^{-7}, were inoculated into HeLa cell cultures, using two tubes for each dilution of inoculum. The results obtained are summarized in Table IV.

As can be seen from Table IV, only the clot from blood taken on the 7th day of illness contained no virus. Serum, also from this blood sample, contained very little virus in that only when it was tested undiluted was virus detectable. Of the tissues obtained at autopsy, all were relatively rich in virus and the higher dilution tubes, including the 10^{-7} dilution, in all instances showed a cytopathic effect. It is noteworthy but puzzling that in cultures of the lung, heart, and spleen, no cytopathic effect was observed in the three lowest dilutions, while in the case of the cultures of kidney, the first five dilutions were negative and a viral effect was seen only in the highest dilutions. In these

TABLE IV

Reisolation of EHD Virus from Deer 1-40 Infected with Virus Grown in HeLa Cell Culture

Material tested	Dilutions	Cytopathic effect in HeLa cell cultures
Muscle	10^{-1} to 10^{-7}	Positive
Liver	10^{-1} to 10^{-7}	Positive
Brain	10^{-1} to 10^{-7}	Positive
Clot	10^{-1} to 10^{-7}	Negative
Serum	Undiluted	Positive
Serum	10^{-1} to 10^{-7}	Negative
Lung	10^{-1} to 10^{-3}	Negative
Lung	10^{-4} to 10^{-7}	Positive
Heart muscle	10^{-1} to 10^{-3}	Negative
Heart muscle	10^{-4} to 10^{-7}	Positive
Kidney	10^{-1} to 10^{-5}	Negative
Kidney	10^{-6} to 10^{-7}	Positive
Spleen	10^{-1} to 10^{-3}	Negative
Spleen	10^{-4} to 10^{-7}	Positive

instances, had virus been tested for only with low dilutions of organ suspensions, its presence would have gone completely undetected so far as the HeLa cell cultures were concerned. Strangely, this prozone effect, in which cytopathogenicity was demonstrable at high but not at low dilutions, was not observed in the cases of tests for virus in suspensions of liver, brain, and gluteal muscle. Neither had it been observed in the case of cultures of the spleen of deer 1-39 used to initiate these studies.

In order to be certain that the cytopathic effect observed in the HeLa cell cultures from the various organs and tissues of deer 1-40 was actually due to EHD virus, the agent derived from gluteal muscle was carried for three further serial passages in HeLa cells before being tested against a known neutralizing serum. The test was conducted using preinfection serum of deer 1-40, serum obtained from deer 1-40 on its 7th day of illness, and serum from deer 1-38

that had recovered from infection with the New Jersey strain of EHD and whose serum was known to be capable of neutralizing EHD virus in deer.

As shown in the results outlined in Table V, the cytopathic agent from the gluteal muscle of deer 1-40 was neutralized completely by the serum of an animal recovered from EHD, deer 1-38, but not by the preinfection or acute phase serum of deer 1-40. It is evident that, so far as Koch's postulates can be fulfilled with a virus, they have been met in the case of EHD virus. This virus has been propagated in HeLa cell culture with a cytopathic effect, the virus from HeLa cell cultures has caused a characteristic, fatal infection in a deer, and from the tissues of the deer the agent has again been propagated in HeLa cells with a cytopathic effect.

TABLE V

Neutralization of Cytopathic Agent Recovered from Gluteal Muscle of Deer 1-40 by EHD Virus–Immune Serum in HeLa Cell Culture

Serum		Virus (3rd HeLa cell passage from deer 1-40)	
Deer No.	Immune status	Titer of virus	Log neutralization index
1-38	Recovered from New Jersey strain EHD virus	$<10^{0}$	>5.5
1-40	Normal—drawn before infection	$10^{-4.5}$	1
1-40	Drawn on 7th day of illness	10^{-7}*	‡
	None, diluent	$10^{-5.5}$	

* Highest dilution tested.

‡ The serum used in this test contained virus (see Table IV); however, it had been heated at 56°C for 30 minutes.

Identity of the Agents Propagated in Newborn Mice and in HeLa Cells.—So far as could be determined by infectivity and immunologic tests, using experimental deer or sera from deer recovered from EHD, both the cytopathic agent propagated in HeLa cell culture and the agent lethally pathogenic for suckling mice are indeed the New Jersey strain of EHD virus. It did, however, seem worthwhile to us to add further supporting evidence that this was so by demonstrating the identity of the mouse-passaged virus with that propagated in HeLa cells. With this purpose in mind, we have utilized the two obvious approaches of determining the transmissibility of the mouse-propagated virus to HeLa cell culture and the HeLa cell–grown virus to suckling mice.

When HeLa cell–propagated virus was administered intracerebrally to newborn mice, these animals developed, on the 5th day, signs of illness similar to those in the original mouse passage line. On the 6th day, the brains of those mice that had not already died were harvested and extracted by the sucrose-

acetone method. The resulting antigen was used in a complement-fixation test with immune deer sera, the sera of mice hyperimmunized with virus of the mouse line, and appropriate controls. The results are presented in Table VI.

As shown by the findings outlined in Table VI, the immune sera tested reacted identically with the antigen produced by virus of the HeLa cell culture line and with that from the mouse passage line.

In an attempt to demonstrate the identity of the mouse- and HeLa cell–passaged viruses in the reverse direction, mouse passage virus was transferred to HeLa cell cultures. The cytopathic effect induced in these cultures was the same as that observed in the original HeLa cell passage line. The titer of the

TABLE VI

Fixation of Complement by EHD Virus–Immune Sera when Tested with Infected Mouse Brain Complement-Fixing Antigen Derived from Either Mouse or HeLa Cell Passage Virus

Serum		Complement-fixing antigen		
Deer No.	Immune to	Brains of mice infected with mouse passage EHD virus	Brains of mice infected with HeLa cell passage EHD virus	Brains of mice infected with unrelated virus
1-38	New Jersey strain EHD virus	64/32*	64/64	0
1-41	New Jersey strain EHD virus	16/32	16/32	0
1-34	Normal	0	0	0
Pooled mice	New Jersey strain EHD virus	128/16	128/16	0

* $\frac{\text{Reciprocal of serum titer}}{\text{Reciprocal of antigen titer}}$

mouse passage virus in the HeLa cell cultures was 10^7 $TCID_{50}$ per 0.1 ml, which permitted the use of infected mouse brain in a neutralization test with sera of EHD virus-immune deer in HeLa cell culture.

As shown in Table VII, the sera of deer recovered from infection with the New Jersey strain of EHD virus neutralized mouse passage virus in HeLa cell culture while the serum of a normal deer did not.

The observations just outlined indicated that both the mouse and HeLa cell passage lines of virus could be propagated in either suckling mice or HeLa cell culture and that each produced identical signs of infection in both systems. Virus from either source was neutralizable in HeLa cell culture by EHD virus–immune serum; furthermore, complement-fixing antigen could be prepared from the brain tissue of suckling mice infected with virus from either source and in each case reacted identically to EHD immune sera. All the evidence that we have been able to develop indicates that the viruses propagated in suckling mice or in HeLa cell culture are the same and are the New Jersey strain of EHD virus.

Sensitivity of EHD Virus to Sodium Desoxycholate.—Nothing is known as to the mode of transmission of EHD virus in nature. In our earlier work (1) we were unsuccessful in transmitting it by pen contact of sick with normal deer. This apparent non-contagiousness of the disease as well as its prevalence in New Jersey in 1955, at a time when biting insects were numerous, suggested that the virus might be arthropod-borne. In order to determine whether or not the EHD virus had the usual sensitivity of the arbor viruses to the action of sodium desoxycholate, a test of this, using Theiler's technique (4), was conducted. The virus suspensions were centrifuged for 1 hour at 10,000 RPM and mixed with an equal quantity of a 1 per cent solution of sodium desoxycholate.

TABLE VII

The Neutralization of Mouse-Propagated Virus in HeLa Cell Culture by the Sera of EHD-Recovered Deer

Serum		Virus (mouse passage)	
Deer No.	Immune to	Titer of virus	Log neutralization index
7-4	New Jersey strain EHD virus	10^{-2}	5
1-38	New Jersey strain EHD virus	10^{-1}	6
1-34	Normal	10^{-7}	0
	None, diluent	10^{-7}	

TABLE VIII

The Sensitivity of the New Jersey Strain of EHD Virus to Sodium Desoxycholate (DCA)

Virus source	Titer of virus	Titer of virus and DCA	Inactivation in logs
Infected HeLa cell culture	$10^{-4.5}$	10^{-4}	0.5
Infected mouse brain	10^{-5}	10^{-4}	1

Following incubation for 1 hour at 37°C, serial tenfold dilutions of the mixture were made and HeLa cell cultures inoculated. Two tubes were inoculated with each dilution and the results are recorded in Table VIII.

As is evident from Table VIII, the New Jersey strain of EHD virus exhibited, although to a limited extent, a sensitivity to sodium desoxycholate, thus fulfilling one of the criteria for inclusion in the arbor virus group.

DISCUSSION

The finding that the New Jersey strain of EHD virus is fatally infectious for newborn mice and is transmissible in HeLa cell culture with a cytopathic effect will make subsequent work with EHD easier and more satisfactory than

it was when deer were employed as the sole susceptible experimental animal. In addition, several features brought out in the present study have yielded information that is not only of interest from the standpoint of EHD but may also have an application in virus disease study in general.

In our initial work with EHD (1) we attempted transmission of the virus in newborn mice on a limited scale and review of our notes in the light of the present findings reveals that there were several late deaths in these first passage mice. However, attempted serial passage of the virus, using the brains of these dead animals, failed. In retrospect, it is apparent that we should have been more alert to relatively minor signs of illness shown by these mice prior to death and should have sacrificed the animals for viral passage before they succumbed. Had we done this, it seems certain that we would have discovered the mouse pathogenicity of EHD virus in our initial work.

What appears to have been another mistake in judgment in our earlier work concerns the choice of the tissue culture system in which to attempt cultivation of the EHD virus. Reasoning that, since we were dealing with a virus of deer, cultures of deer kidney cells should afford the best system in which to attempt cultivation, we tried such cultures and failed to obtain growth of the virus. This failure in what we had thought to be the optimal system deterred us from further work with tissue cultures at the time and only after the success of one of us (2) in growing another virus causing a hemorrhagic type of disease did HeLa cell cultures appear worthy of a trial with EHD virus. Our success in growing a virus from deer in HeLa cell cultures perhaps emphasizes the fallacy of being too logical in the selection of a tissue culture system in which to attempt the cultivation of a new virus.

Another feature of the current study that may have virological implications beyond EHD concerns the finding that, in attempting growth of the virus from certain deer tissues, only the higher dilutions tested yielded detectable virus. Had suspensions of lung, heart muscle, or spleen from the deer that died of HeLa cell-propagated virus not been tested in dilutions beyond 10^{-3} or kidney in dilutions beyond 10^{-5}, virus would not have been detected. The reasons for this failure to obtain a cytopathic effect at lower dilutions, even though at higher dilutions the virus was cytopathic, are not clear. Though this particular deer survived for 9 days after the onset of its illness, it seems doubtful that virus-neutralizing antibody could have been formed in this period of time to account for the viral inhibition in the tissues in which it was evident. Also failure to observe the inhibitory effect in low dilutions of suspensions of brain, gluteal muscle, and liver makes virus-neutralizing antibody seem an unlikely explanation of the inhibitory effect. At any rate, whatever the explanation, the finding rather emphasizes the importance, when testing for the presence of virus in organ suspensions, of not limiting the tests to low dilutions. Though high concentrations of virus were present in the organs of

the deer under discussion, the presence of virus in several of them would quite obviously have been missed had not high dilutions of the suspensions been tested in the tissue culture system.

Only the New Jersey strain of EHD has been propagated in newborn mice and in HeLa cell culture and tests of its relationship to the South Dakota strain with the more quantitative immunological tests now possible in these new systems have been made only in one direction. It has been found that, with complement-fixing antigen made from the brains of mice infected with New Jersey strain virus, sera from deer immune to either the New Jersey or South Dakota virus fix complement about equally well. Although in the absence of knowledge of the homologous titers of the South Dakota immune sera no definitive statement can be made, it may tentatively be concluded that the complement fixation test probably is not capable of differentiating between the two strains of virus. Also the virus neutralization test in HeLa cell culture, though showing a quantitative difference in the neutralizability of New Jersey strain EHD virus by sera of deer immune to the South Dakota or New Jersey strain of virus, does not yield a clear-cut answer as to whether these two strains are actually serologically different as they appeared to be in our original cross-immunity tests in deer (1). Only when we have succeeded in propagating the South Dakota strain of EHD virus in tissue culture and can then make a quantitative serological comparison with the New Jersey strain can a decision be reached as to the antigenic relationship existing between the two.

SUMMARY

The New Jersey strain of EHD virus has been propagated in newborn Swiss mice by the intracerebral route and is regularly lethal beyond the first serial mouse passage. A complement-fixing antigen prepared from the brains of infected mice reacts positively with the sera of deer recovered from infection with either the New Jersey or South Dakota strain of virus, but not with the serum of normal deer. The mouse-passaged virus induced an inapparent infection in an experimental deer.

The virus can also be grown serially in HeLa cell culture and induces a characteristic cytopathic effect. It is neutralizable in such cultures to high titer by the sera of deer recovered from EHD (New Jersey strain) and to lower titer by the serum of a deer recovered from EHD (South Dakota strain). Normal deer serum does not neutralize the virus in tissue culture. The HeLa cell–passaged virus induced typical lethal EHD in an experimental deer and virus could be recovered from most of the tissues of this animal in HeLa cell culture. An unexplained prozone of inhibition of cytopathogenicity at low dilutions was observed in cultures of some of the organs.

The fact that EHD virus exhibited a limited sensitivity to sodium desoxycholate suggests that it may belong in the arbor virus group.

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