Cultivation and Modification of Infectious Canine Hepatitis Virus in Roller Tube Cultures of Dog Kidney. (21242)

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The observation that multiplication of poliomyelitis viruses in tissue culture is accompanied by certain cytologic changes which are inhibited specifically by immune serum (1) has opened a broad field of study for the further application of tissue culture technics. In the study of certain highly host specific virus diseases of domestic animals research has been limited by the expense and necessity of providing both susceptible animals and suitable isolation quarters. Since infectious canine hepatitis (ICH) was described by Rubarth(2), investigations in this country have shown the disease to be of major importance among the canine population. Diagnosis is often difficult, since most frequently the only signs of disease are highly elevated temperature and leukopenia. Although the disease terminates fatally in only about 10% of the cases, there is a high carrier rate among recovered animals, the virus being excreted in the urine for as long as 161 days after infection(3). While recovery from infection results in lifelong immunity, immunization with the available inactivated tissue vaccines results in only a short-lived immunity.

It is the purpose of this paper to describe the cultivation and modification of ICH virus and a quantitative neutralization test for the diagnosis of this disease.

Materials and methods. Tissue cultures. Roller tube cultures were used throughout these experiments although the method of preparation varied. Up to the 48th passage of ICH, cultures of kidney explants were prepared by the plasma clot method described by Enders and his associates(4,5). Subsequent cultures were prepared by the simpler method of trypsinization of the minced kidney cortex used by Dulbecco and Vogt(6). A 0.2% suspension of washed cells was made in the nutrient fluid, which consisted of 8 parts Earle's-Simms solution, one part 5% lactalbumin, one part inactivated horse serum. Two ml of this suspension adjusted to pH 7.6-7.8 were placed in roller tubes and incubated stationary at 35°C until solid sheets of epithelial cells had formed on the walls of the tubes. The nutrient fluids were then renewed and the tubes were inoculated and placed in roller drums for further incubation. For the initial experiments kidneys were obtained from 2-4 week old pigs and for the remainder from 2-4 week old puppies from ICH and distemper immune bitches. All dogs used for virus inoculation were 2-5 months of age and were obtained from ICH susceptible litters.

Experimental. Propagation of the virus. During the course of experiments involving the use of pig kidney cultures, attempts were made to adapt ICH virus to this tissue. A 20% suspension of dog liver infected with ICH was inoculated into each of several tubes containing pig kidney explants. Serial transfers of 0.2 ml undiluted tissue culture fluid were made at 4-day intervals. No effects of the virus on the cells were noted in any tubes at any passage level. However, the 5th passage material when diluted 10^{-3} and inoculated in 0.2 ml amounts subcutaneously in 2 dogs resulted in the death of one animal with typical gross pathological lesions of ICH and a febrile response in the other dog. This animal, on recovery, was found to be immune to challenge with the original infected dog liver material. This tissue culture fluid represented a dilution of $10^{-8.7}$ of the original material which had a titer in dogs of only $10^{-6.5}$ when one ml was injected intravenously. At the 7th passage level in pig kidney the undiluted fluid inoculated into the 2 dogs resulted in the death of both. However, at the 8th passage ICH virus was no longer present. Cultures were then prepared from spleen and whole kidney of 2-week-old puppies. These were inoculated with 0.2 ml of undiluted fluid from the 3rd pig kidney passage. On the 6th day of inoculation only the epithelial cells in kidney cultures showed marked cytologic changes. The cells were rounded and highly refractile.

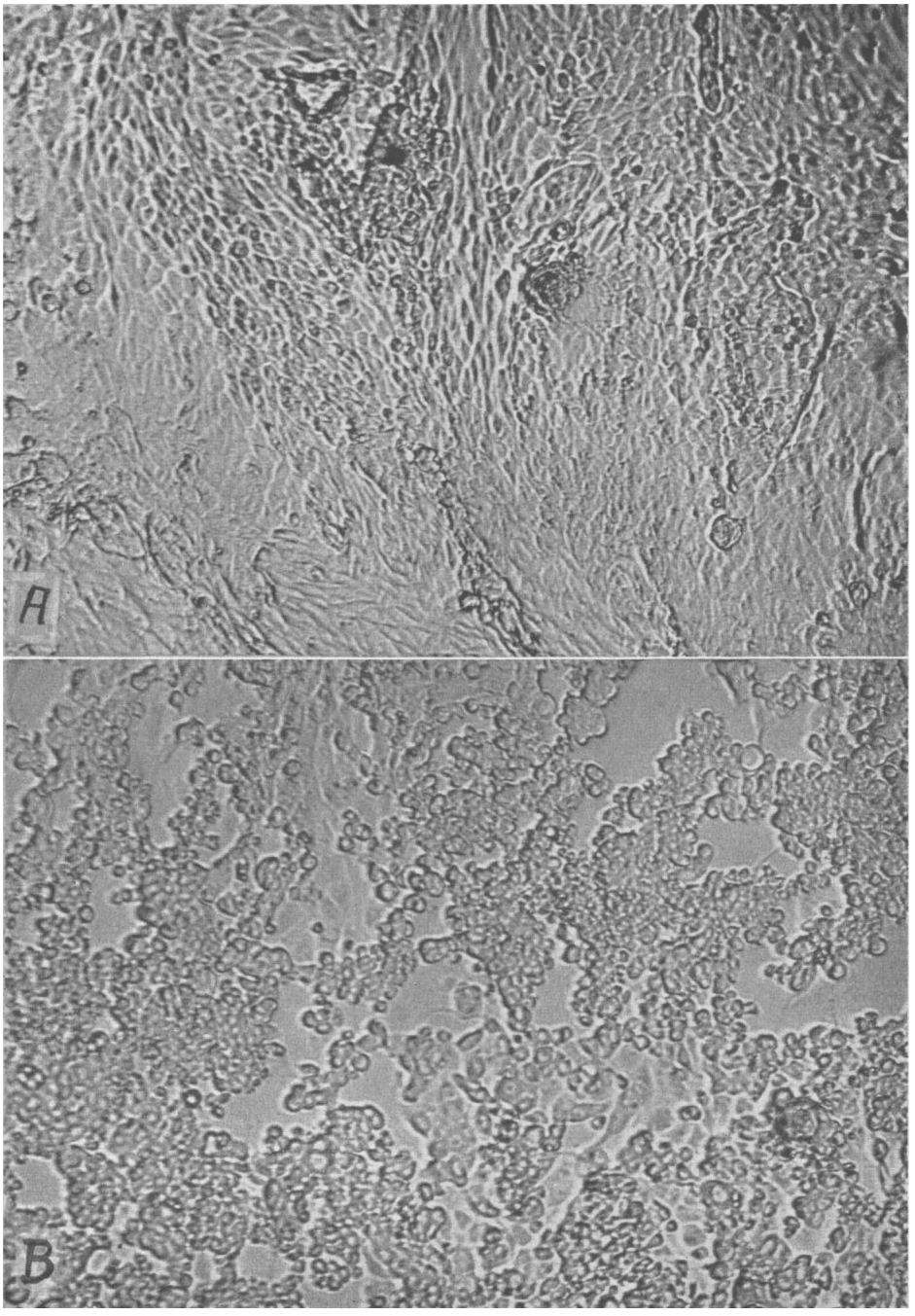

FIG. 1. Effect of ICH virus on roller tube cultures of dog kidney. (A) Uninoculated control tube showing clear sheet of epithelial cells. (B) Two days after inoculation with 10^4 $TCID_{50}$ of ICH virus, cytologic changes are evident.

The epithelial sheets had broken up and the cells were in small grape-like clusters. The fibroblasts in the same cultures were unaffected and continued to grow even though all epithelial cells showed cytologic changes. The spleen cultures which contained only

fibroblasts remained normal throughout the 2-week observation period. The supernatant fluids from the kidney cultures were removed and serial passage of 0.2 ml of undiluted fluid was carried out. On subsequent passages the cytopathogenic effect occurred after 24-48 hours of incubation. Passages were made at 2-3 day intervals. The photomicrographs in Fig. 1 illustrate the effect of ICH virus on dog kidney cultures as seen 2 days after inoculation with undiluted tissue culture fluid.

Identification of the tissue culture virus was made at the 10th passage, at which time the titer of the virus was $10^{-5.6}$. Hyperimmune serum was prepared by inoculating a puppy with ICH virus known to be free of contamination. The dog developed a strong febrile reaction and 2 more injections of this virus were made 2 and 3 weeks after the original inoculation. The dog was bled and serum removed for neutralization tests. The serum had a neutralization index of 250,000+ and a 50% serum dilution endpoint of 1:4,096 against 400 tissue culture doses of virus, thus establishing the identity of the tissue culture virus with that of the original ICH virus.

Neutralization tests. At the 11th tissue culture passage a large pool of virus was prepared which had a titer of $10^{-6.2}$ in tissue culture and $10^{-7.5}$ in dogs. However, it was obvious at this time that a febrile reaction in dogs as an indication of ICH infection was unreliable, since any number of intercurrent infections might also give rise to a febrile reaction. The determination of antibody by the quantitative neutralization test offered a positive means of diagnosis and at the same time a method for testing the susceptibility of each dog in a litter before use in a test. All dogs were bled by cardiac puncture before inoculation and 2 and 3 weeks after inoculation. Decimal dilutions of sera were made in the tissue culture nutrient fluid, each dilution being mixed with an equal quantity of virus so that 0.2 ml of serum-virus mixture contained 100-320 $TCID_{50}$ of ICH virus. Incubation was carried out for 2 hours at 37°C after which time the 0.2 ml of each mixture was inoculated into each of 4 tubes. No sera were tested undiluted since it had been found that a large percentage of young dogs carried passively transferred neutralizing antibody to ICH, insufficient to protect against the disease. All sera from each dog were tested simultaneously. Final readings were made at 5-6 days when the control titration showed 100-320 $TCID_{50}$ of virus to be present.

TABLE I. Titration of Tissue Culture ICH Virus in 12 Dogs with Results of Serum Neutralization Tests.

Virus dilution	Clinical response	Reciprocal of 50% serum dilution endpoint against 100-320 $TCID_{50}$ of ICH virus (pre-inoc. titer 1:4) 2 wk convalescent	3 wk convalescent
10^{-2}	F_4	3160	3160
	F_5	316	3160
10^{-3}	F_4	2160	10000
	"	3160	4650
10^{-4}	"	2150	3200
	F_5	3200	3200
10^{-5}	F_3	3200	3200
	F_4	1000	21500
10^{-6}	"	215	1000
	"	2150	3200
10^{-7}	F_6 D_{12}	—	—
	F_6	3200	3200

F = Onset of febrile reaction on designated day.
D = Died 12 days post inoculation.
* Virus inoculated subcut. in 0.2 ml amounts.

In Table I are summarized the data of a typical titration of ICH virus in dogs with the results of serum neutralization tests.

Although no endpoint was attained in this titration it is apparent that the dogs were highly susceptible to infection with the tissue culture virus and that high levels of antibody were reached as early as 8 days after the febrile response was first noted. The value of the neutralization tests for diagnostic purposes was emphasized by the fact that signs pathognomonic of the disease failed to develop in these dogs. Although the febrile reactions ranged from 39.8°C to 40.7°C they alone would not be sufficient to establish a diagnosis of ICH.

Modification of the virus. Serial passage of ICH was maintained over a period of several months. Occasional inoculation of dogs with undiluted virus was carried out to test for virulence and to check on the specificity of the cytopathogenic effect. Each of 30 dogs inoculated with virus from various tissue culture passages up to the 40th developed signs

TABLE II. Results of Passage of ICH Virus in Tissue Culture Showing Apparent Loss of Virulence for Dogs without Loss of Antigenicity.

Virus inoculum*	No. of dogs inoculated	No. showing clinical signs: Fever	Corneal opacity	% mortality	No. showing rise in neutralizing antibody titers†
TC #10 to 40	30	30	5	24§	23
TC #51 to 70	13‡	1	0	0	13

* All dogs were inoculated subcut. with 0.2 ml containing 10^5 to 10^6 $TCID_{50}$ of virus.

† All sera had pre-inoculation titers of <1:4 and all showed at least 100-fold rise in antibody titer.

‡ Litter mates of these dogs when inoculated with virulent ICH virus developed definite signs of disease.

§ Diagnosis of ICH established post mortem either by presence of intranuclear inclusions or recovery of virus from liver and typical gross pathological lesions of ICH.

of disease. Only one of 13 dogs inoculated with virus from the 51st to 70th passages developed a slight febrile reaction. All dogs which had recovered from infection by the tissue culture virus were routinely challenged with the original virus from dogs and found to be solidly immune. Dogs inoculated with virus from the 51st to 70th tissue culture passages and challenged one month after inoculation were also refractory. It is conceivable, however, that these dogs were immune prior to inoculation with the tissue culture virus, even though litter mates had been highly susceptible. Neutralization tests were therefore utilized to establish the immune status of these animals. In Table II are summarized the data obtained by inoculation with virus from various tissue culture passages. The data suggest that the virus underwent modification in its virulence without loss of ability to stimulate antibody formation.

Discussion. Numerous attempts in this laboratory to adapt ICH virus to chick embryos, suckling mice, ferrets and rabbits have been without success. Although there have been reports of successful adaptation to chick embryos(7), ferrets(8), and rabbits(9), these results remain to be confirmed. The cultivation of ICH virus in cultures of dog kidneys has been reported recently by Cabasso *et al.* (10).

In order to avoid supply and maintenance of large numbers of susceptible puppies in isolation quarters, attempts were made to cultivate the virus in the tissues of the natural host. The virus proved readily adaptable to growth in kidney explants and also produced readily recognizable cytologic changes. Furthermore, it was found that kidney cortex from 2-4 week old puppies had an extremely fast rate of growth, producing large sheets of epithelial cells in 3 to 4 days, in contrast to the slow rate of growth in cultures from older dogs. Since the puppies were obtained from bitches which had been recently hyperimmunized against ICH and canine distemper, the danger of accidental contamination with, and subsequent passage of, either of these viruses was minimized. In addition, kidneys from dogs of this age could be trypsinized more readily than those from older dogs. More than 400 tubes have been made from one pair of kidneys, all of which showed good, uniform growth.

The neutralization test in tissue culture has proven to be a most useful and sensitive diagnostic aid. All of 30 dogs whose sera had a pre-inoculation titer of 1:4 or less were shown to be susceptible to infection as indicated both by signs of disease and development of high titers of neutralizing antibody. On the other hand, 10 dogs which had pre-inoculation titers ranging from 1:1000-1:32000 were completely resistant to infection.

Up to the 40th passage of ICH the virus was highly virulent, producing a febrile reaction in all dogs inoculated, corneal opacity in 37% and death in 24%. However, at the 51st passage and through the 70th, the virus appeared to undergo a striking change in virulence. It no longer seemed capable of causing death or corneal opacity and produced a febrile reaction in only one dog. The immunizing properties of the virus remained un-

changed. At this point the value of the neutralization test for diagnostic purposes was further emphasized. After modification had occurred it was possible only by demonstrating a rise in antibody titer to prove that the dogs had been infected. However, diagnosis of ICH has also been made on numerous occasions by direct isolations of the virus in tissue cultures, either from a single drop of blood obtained during the febrile period; or after death from the livers of infected dogs(11). Thus, as has already been established for poliomyelitis, diagnosis can be made either directly by virus isolation or indirectly by demonstrating increase in antibody titer.

Recently, a comparison was made between virus from the 74th tissue culture passage and virulent ICH virus. There were 6 dogs in each group with an equal distribution of litter mates in each group. None of the dogs inoculated with virus from the 74th tissue culture passage developed either a febrile reaction or a leukopenia. Five of the dogs inoculated with virulent ICH virus died and had typical post mortem lesions of ICH, while the 6th dog developed a strong febrile reaction. All of the vaccinates developed a high titer of neutralizing antibody and resisted challenge with the virulent virus.

Summary. 1. Propagation of ICH virus with the production of distinctive cytologic changes has been demonstrated in roller tube cultures of dog kidney. 2. After 51 serial passages the virus apparently had lost its virulence for dogs, although producing a solid immunity. 3. A practical neutralization test for the diagnosis of ICH has been described.

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Effects of Tumbling Trauma, Scalding and Hemorrhage on Rat Tissue Non-Protein Sulfhydryl.* (21243)

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In a preceding publication(1), 2 of us have reported that scalding, tourniquet trauma and exposure to severe cold for 6 hours all induced in the mouse a marked decrease in liver non-protein sulfhydryl (–SH) concentration. The present paper deals with the effects in the rat of tumbling trauma and scalding on non-protein –SH concentration in the blood, liver, and certain other tissues. Data on liver –SH values obtained for rats subjected to severe hemorrhage are also presented.

Materials and methods. Chemical estimations. All distilled water employed in the present experiments was freed of heavy metal ions capable of binding –SH by passing ordinary distilled water through a La Motte Filtrion column. –SH estimations for deprotein-

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