Propagation of Infectious Canine Hepatitis Virus in Cultures of Pig and Ferret Kidney. (23329)

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Propagation of infectious canine hepatitis virus (ICH) in cultures of canine renal epithelium has been reported(1,2). After 41 serial passages the virus became modified in its virulence for dogs(2), but retained its *in vivo* renal tropism. Even after 86 passages in kidney cultures plus 10 passages in canine uterus cultures all dogs that were tested excreted modified virus in the urine(3). Susceptible dogs placed in contact with vaccinated dogs developed an immunity to ICH without showing signs of disease. These also excreted modified virus. It was possible to produce inapparent infection by this method by 4 successive serial back-passages in dogs, but at the fifth passage the virus reverted to virulence and 2 of 3 exposed dogs developed a fatal ICH infection.

It seemed likely that if the carrier state were to be eliminated the virus would have to be propagated in tissues of other animals. Although ICH virus naturally infects members of the canine species only, it has been reported that corneal opacity typical of this disease could be produced in ferrets inoculated intraocularly with ICH virus(1). In our initial experiments equivocal evidence was obtained that unadapted ICH virus from canine tissues multiplied at a very low level in pig kidney cultures(2). Both of these observations seemed to warrant further investigation. We present evidence that ICH virus can be propagated in cultures of ferret and pig kidney with the production of consistent and typical cytological changes.

Materials and methods. Tissue cultures. The pig and dog kidney cultures were continuous lines of epithelial-like cells derived from cells cultivated originally by the method of Youngner(4). Stock cultures were carried in 2 and 5 l. Povitsky bottles. Cell suspensions were made by replacing the medium with an equal quantity of buffered solution of 1:5000 versene (disodium salt of ethylene-diaminetetracetate). After incubation at 36°C for 15-30 minutes, with occasional shaking, the cells peeled from the glass, and formed a homogeneous single cell suspension. The cells were centrifuged at 1,000 rpm for 5 minutes, the versene discarded and the cells resuspended in growth medium equivalent to twice the original volume. *Pig kidney* cells for virus propagation were distributed in 6 ml amounts to 2 oz. French squares or prescription bottles. Occasionally 8 oz. Blake bottles containing 50 ml of cell suspensions were used for cultivation. A complete monolayer of cells generally formed in 7-9 days. *Dog kidney* cells were used for virus titrations only and distributed in 2 ml amounts to 16 x 150 mm tubes. These cells had an extremely fast rate of growth, forming a complete sheet in 2-3 days; multiplication being observed after 8 hours of incubation at 36°C. *Ferret kidney* cultures were primary cultures of epithelial-like cells prepared from adult animals by the method of Youngner. Both roller tubes and bottle cultures were utilized. Otherwise maintenance of cultures and cultivation of virus was the same as for pig and dog kidney. *Media.* Growth media for all cells contained 0.5% lactalbumin hydrolysate and 0.2% yeast extract in Earle's balanced salt solution. However, the latter was modified by decreasing the $NaHCO_3$ content by 50% and adding 1.2 g/l of tris (hydroxymethyl) aminomethane(5). The pH was adjusted to 7.4 by addition of 1 N HCl. For cultivation of pig kidney cells the growth medium was supplemented with either pig or calf serum in final concentration of 20%. The dog kidney cells required only 5% calf, lamb or horse serum in addition to the growth media, while the ferret kidney grew best with a supplement of 20% lamb serum. After the cultures had

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formed complete monolayers, unless they were used immediately for virus propagation, serum concentration was maintained at 5%. For propagation of ICH virus all cultures were treated in the same manner. The growth medium was removed and replaced with equal quantity of phosphate buffered saline (PBS) which bathed the cells for 1 hour at 36°C. This was done to remove antiviral substances which might have been present in the serum. The PBS was then replaced by equal quantity of medium 199(6), pH 7.6. Serum was not used during virus growth phase and cultures could be maintained for periods to 2 weeks at 36°C. All media contained 200 units penicillin and 200 μg streptomycin/ml.

Virus titrations. The amount of virus obtained from both pig and ferret cultures was determined by inoculation of decimal dilutions of infected fluids into roller tube cultures of dog kidney. Dilutions were prepared in medium 199 and 0.2 ml aliquots of each dilution were added to 4 tubes containing 1.8 ml of media. Cultures were rotated at 36°C and examined for characteristic cytologic changes daily for 10 days. Titers are expressed as number of tissue culture infectious doses/ml ($TCID_{50}$) as calculated by the method of Reed and Muench(7).

Results. Propagation of ICH virus in ferret kidney. Although virus obtained from different passage levels in dog kidney was propagated in ferret kidney, only the experiments carried out with virus from the 12th dog kidney passage will be reported. In the first passage in ferret kidney 1 ml of virus was inoculated into 8 oz. Blake bottles. Cytologic changes were observed after 48 hours, involving all cells by 72 hours. A second passage was made in similar manner from harvested fluids, but cytologic changes occurred slowly and involved only about 50% of the cells by the 7th day. Subsequent passages were made by inoculating undiluted culture fluid into roller tubes or French squares to give a final dilution of 1:10. Rarely was more than half the cell population affected. In 2 instances non-specific degeneration occurred after 14 days, before specific cytolysis was noted. It was necessary to go back to the previous passage and use the infected fluid as the sole source of both virus and nutrient fluid. This was done with both the 7th and 12th passages and it will be noted from Table I that harvest of these fluids was carried out at 10 and 4 days respectively when at least 50% of the cells showed specific changes. Although virus yields were greater in dog kidney than in ferret kidney there is little doubt that the virus propagated in the latter. Virus present in the 18th ferret passage represented a cumulative dilution of the original inoculum of $10^{-22.6}$.

TABLE I. Results of Serial Passage of ICH Virus in Ferret Kidney Cultures.

Passage No.		Harvest (day after inoc.)	−Log of titer/ml
DK*	12		8.0
FK†	1	3	7.0
	2	7	6.0
	3	5	6.2
	4	5	5.7
	5	7	4.4
	6‡	8	N.D.
	7	10	N.D.
	8	8	5.0
	9	7	4.4
	10	8	4.5
	11‡	9	5.0
	12	4	6.2+
	13	4	N.D.
	14	7	5.0
	15	6	5.4
	16	8	4.9
	17	6	4.7
	18	11	5.2

* Dog kidney. † Ferret kidney. N.D., not done.

‡ Harvested fluids from these passages were used as sole source of nutrient and virus inoculum for succeeding passage. In all other passages final dilution of inoculum was 1:10 in fresh nutrient fluid.

Propagation of ICH virus in pig kidney cultures. Cytologic changes invariably occurred 2-3 days after inoculation of undiluted virus into bottle cultures where final dilution was 1:50 in the initial passage and 1:10 in each of the following passages. Although the changes observed were different from those observed in either the dog or ferret kidney they were readily recognizable and characteristic of ICH virus in this tissue. Fluids from each passage were harvested when 50 to 100% of the cells showed signs of infection which was generally 1 to 4 days after initial observation of cytologic changes. The results of 11 serial passages of ICH virus in pig kidney are summarized in Table II. While the

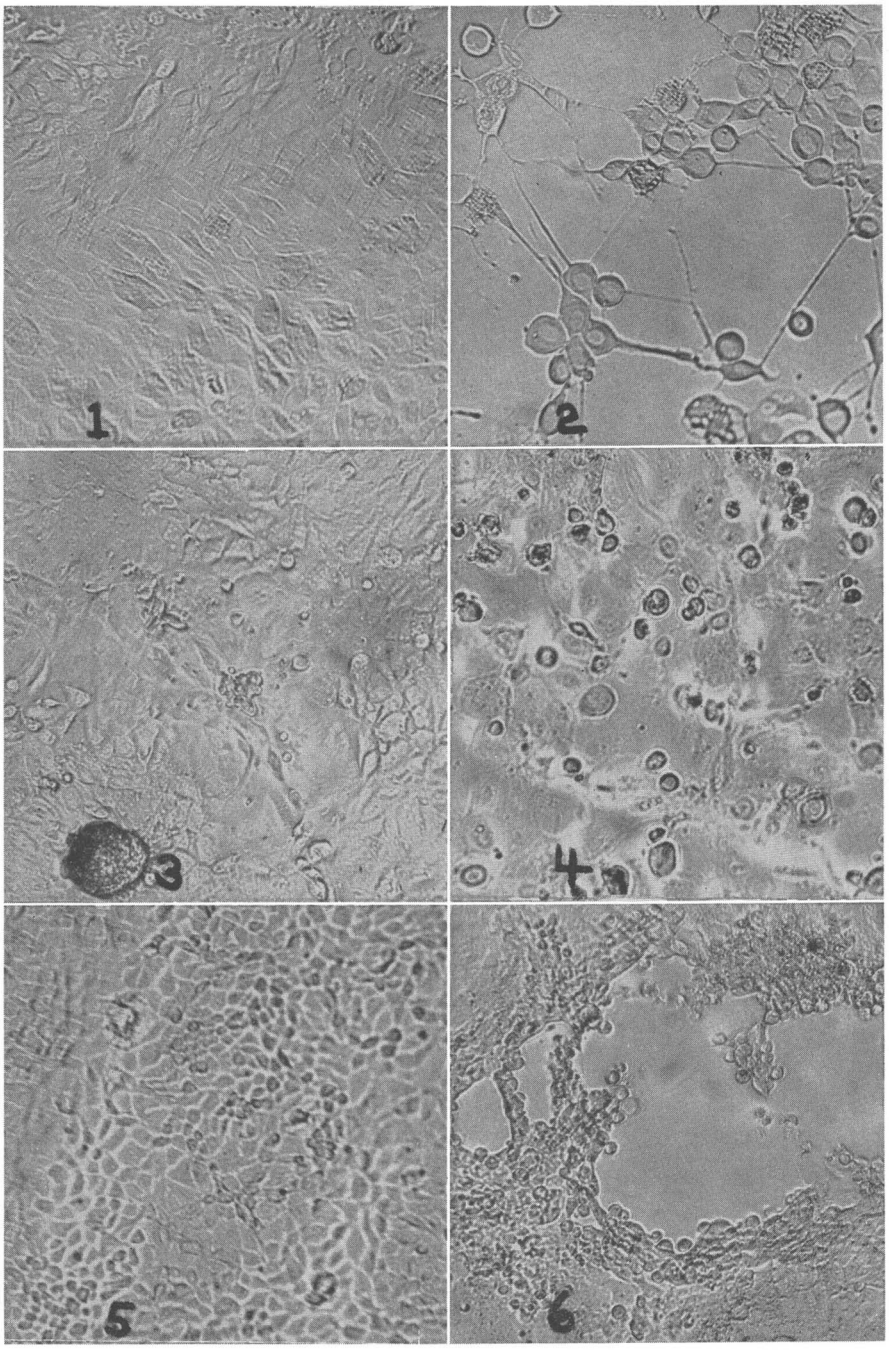

FIG. 1. Continuous culture of normal dog kidney.

FIG. 2. Cytologic changes in dog kidney culture 3 days after inoculation with 10^6 $TCID_{50}$ of ICH virus.

FIG. 3. Primary culture of normal ferret kidney.

FIG. 4. Cytologic changes in ferret kidney 6 days after inoculation with 10^5 $TCID_{50}$ of ICH virus.

FIG. 5. Continuous culture of normal pig kidney.

FIG. 6. Cytologic changes in pig kidney 4 days after inoculation with 10^5 $TCID_{50}$ of ICH virus. (All figures 100 ×.)

TABLE II. Results of Serial Passage of ICH Virus in Pig Kidney Cultures.

Passage No.		Harvest (day after inoc.)	−Log of titer/ml
DK*	12		8.0
PK†	1	5	N.D.
	2	7	5.2
	3	6	5.7
	4	4	N.D.
	5	4	5.4
	6	4	6.2
	7	6	7.0
	8	4	7.2
	9	4	6.7
	10	3	6.0
	11	7	7.2

* Dog kidney. † Pig kidney. N.D., not done.

titers are higher than those obtained from virus passaged in ferret kidney, they are not as high as those obtained from dog kidney passaged virus. However, they are consistent with virus multiplication since fluids harvested from the 11th passage represented theoretical virus activity at cumulative dilution of the original inoculum of $10^{-18.1}$.

The ready adaptability of ICH virus to continuous cultures of pig kidney was demonstrated in the line of virus which had been passaged both in dog and ferret kidney. Virus from the 13th ferret kidney passage was passed serially in pig kidney cultures and consistently produced cytologic changes in 2 to 3 days with high titers of virus by 3 to 7 days after inoculation (Table III). Virus activity from the 10th passage of this series represented a cumulative dilution from the original dog kidney virus of $10^{-29.1}$. For comparative purposes cytologic changes produced by ICH virus in dog, ferret and pig kidney are illustrated in Fig. 1-6. Control cultures are of the same age as the inoculated ones and received the same treatment with the exception of virus inoculations. In no instance were similar cytologic changes noted in control cultures.

TABLE III. Results of Passage of Ferret Kidney Adapted ICH Virus in Cultures of Pig Kidney.

Passage No.		Harvest (day after inoc.)	−Log of titer/ml
DK*	12		N.D.
FK†	13		N.D.
PK‡	1	7	5.7
	2	6	5.7
	3	4	N.D.
	4	4	6.2
	5	4	6.2
	6	6	7.0
	7	4	6.7
	8	4	6.5
	9	3	7.2
	10	7	7.2

* Dog kidney. † Ferret kidney. ‡ Pig kidney. N.D., not done.

Identification of the 3 lines of passage viruses was established by neutralization tests carried out in dog kidney cultures. Pooled serum from dogs known to be susceptible to ICH virus failed to neutralize any of the passage viruses. However, serum from these same dogs obtained after they had undergone active infection produced by known ICH virus neutralized each of the 3 passage viruses. Furthermore, 4 dogs which were inoculated with ferret kidney passaged virus developed antibodies against known dog kidney passaged virus and resisted challenge with virulent ICH virus.

The work reported here has increased the host range of ICH virus to tissues of species totally unrelated to the dog. However, it remains to be determined if on continuous serial passage in either or both ferret and pig kidney the virus will lose its *in vivo* renal tropism in dogs so that it may be used safely in modified form as an immunizing agent.

Summary. Infectious canine hepatitis virus, a hitherto highly host specific virus has been propagated in primary cultures of ferret kidney and in continuous cultures of pig kidney, virus multiplication being accompanied by distinctive cytologic changes. The virus was identified by neutralization tests with specific antiserum.

1. Cabasso, V. J., Stebbins, M. R., Norton, I. W., and Cox, H. R., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 239.

2. Fieldsteel, A. H., and Emery, J. B., *ibid.*, 1954, v86, 819.

3. ———, *Am. J. Vet. Res.*, 1956, v17, 380.

4. Youngner, J. S., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 202.

5. Swim, H. E., and Parker, R. F., *Science*, 1955, v122, 466.

6. Morgan, J. F., Morton, H. J., and Parker, R. C., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 1.

7. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, v27, 493.

Received May 23, 1957. P.S.E.B.M., 1957, v95.