# Propagation of Mouse Hepatitis Virus (Gledhill) in Tissue Culture. (24536)

THEODORE J. STARR* AND MORRIS POLLARD

*University of Texas Medical Branch, Dept. of Preventive Medicine and Public Health, Virus Research Lab., Galveston, Texas*

The Gledhill strain of mouse hepatitis virus (MHV 1)(1,2) causes a mild, rarely fatal infection in weanling Swiss mice and a fatal infection in newborn mice. Host-specific viruses associated with hepatitis infections of duck(3-5), dog(6), and man(7) have also been described. However, only agents of canine hepatitis and of Buescher's mouse hepatitis(8) have been propagated *in vitro*(9-12). This communication describes procedures for propagation of MHV 1 in tissue cultures of mouse kidney explants. As MHV 1 did not cause consistent cytopathogenic effects in our tissue cultures, infectivity tests with newborn mice were used to detect the fate of this virus in serially passaged cultures.

*Materials and methods. Stock virus.* A 5 g pool of livers from suckling Swiss mice, which as newborns were infected 5 days previously *via* the peritoneal route, was homogenized in a Ten Broeck grinder with Hank's balanced salt solution (BSS) (10% w/v) containing streptomycin (0.2 mg/ml) and penicillin (200 units/ml). The chilled homogenate was centrifuged at 2,000 rpm for 30 minutes at 4°C. The partially clarified virus suspension was sealed in ampoules and stored in a dry-ice chest. *Virus titrations.* Serial decimal dilutions of stock virus were prepared in phosphate-buffered saline (PBS) (pH 7.2). Each dilution was inoculated (0.05 ml) *via* peritoneal route into a litter of 1-2 day old Swiss mice (5-8 mice/litter). Five days later surviving mice were sacrificed with ether. Liver damage was noted and the 50% infective dose ($ID_{50}$)/0.05 ml of inoculum was calculated(13). Grossly affected livers were removed aseptically. They were frozen immediately and subsequently used for preparation of stock virus. Stock virus prepared and assayed by these methods had $ID_{50}$ endpoints of $10^{-3.3}$ to $10^{-3.6}$. Attempts to achieve higher titres either by repeated freezing and thawing or by repeated extraction of liver tissues with PBS or BSS failed. *Tissue culture media.* The following media used for growth of kidney explant cultures and for virus propagation contained streptomycin (0.2 mg/ml) and penicillin (200 units/ml) and were adjusted to pH 7.6-7.8 with a 2.8% solution of sterile sodium bicarbonate: medium A, 5 parts mouse embryo extract (40% stock solution made in BSS), 10 parts LAH (5% stock solution lactalbumen hydrolysate), and 85 parts medium 199; medium B, 10 parts calf, beef, horse, or human serum, 10 parts LAH, and 80 parts medium 199. *Kidney explant cultures.* Kidneys of newborn Swiss mice were removed aseptically and washed twice in BSS, minced finely, rewashed, and suspended in BSS. The kidney mince was dispersed evenly over lower half of screw-cap test tubes (16 x 125 mm) containing a thin coating of chicken plasma and excess fluid was removed. Twenty-five minutes later 1 ml of medium A was added. The cultures were incubated at 37°C on roller drum apparatus (6-8 rph). Those cultures which showed proliferation within 18 hours and confluent sheets of cells within 120 hours were used for virus inoculation. Minced kidneys of 25 newborn mice yielded sufficient tissue for 16-25 explant cultures.

*Results. Attempts to propagate MHV 1.* Propagation of MHV 1 in the following established cell lines was unsuccessful: L strain of mouse fibroblast(14), human amnion(15), HeLa(16), and also commercially available trypsinized monkey kidney (Microbiological Asso.). Virus could not be detected either by cytopathogenic effects or by infectivity trials in newborn mice with cultures assayed from 1-22 days after inoculation. In most instances the virus disappeared within 12-24 hours and did not reappear after 2 or 3 serial passages. In control tubes with medium A

* This investigation supported by James W. McLaughlin Fellowship Fund.

TABLE I. Serial Passage of MHV 1 in Tissue Cultures of Mouse Kidney Explants.

| Passage | Virus dilution | Growth of explants (hr)* | Theoretical† $-\log ID_{50}$ at 0 hr | Calculated $-\log ID_{50}$ | | |
|---|---|---|---|---|---|---|
| | | | | 0 hr | 48 hr | 96 hr |
| 1 | $10^{1}$ | 18 | 2.5 | 2.4 | 4.8 | |
| 2 | $10^{3}$ | 18 | 2.8 | 3.0 | 4.6 | |
| 3 | $10^{4}$ | 72 | 3.6 | 2.5 | | 4.8 |
| 4 | $10^{5}$ | 120 | 3.8 | 3.8 | | 5.2 |
| 5 | $10^{7}$ | 120 | 3.2 | 1.9 | | 4.4 |
| 6 | $10^{8}$ | 48 | 3.4 | NT‡ | | 4.7 |
| 7 | $10^{9}$ | 48 | 3.7 | 3.7 | | 4.1 |
| 8 | $10^{10}$ | 72 | 3.1 | 3.5 | | 4.8 |
| 9 | $10^{11}$ | 72 | 3.8 | 3.6 | | 4.2 |
| 10 | $10^{12}$ | 48 | 3.2 | 2.7 | | 4.3 |

* Mouse kidney explants were grown for indicated period in medium A (see text) prior to inoculation with stock virus ($ID_{50}$ $10^{-3.5}$) and subsequently with successive passages.

† Theoretical $ID_{50}$ endpoints were 0.1 of calculated titres of respective preceding cultures in all cases except passages 2 and 5 where titres were 0.01 of respective preceding cultures.

‡ Not tested.

and without tissue, the $ID_{50}$ endpoint decreased from $10^{-2.5}$ to $10^{-40.6}$ after 6 hours. The presence of serums (medium B with horse or calf serum) delayed virus decay slightly. In other experiments, stock virus was inoculated into stationary flasks containing medium B with horse serum plus either minced kidneys or livers from newborn mice. Virus disappeared from liver cultures within 24-48 hours and from kidney cultures within 120 hours. Similar results were reported by Pollard(17) who demonstrated the effectiveness of minced kidney tissue for prolonged survival of PRI mouse hepatitis virus(18).

*Serial passage of MHV 1 in mouse kidney explant cultures.* These cultures were prepared with medium A for serial passage of MHV 1 (Table I). After 18-120 hours, nutrient fluids were removed from proliferating explant cultures. Cultures were inoculated with virus and reincubated. The original inoculum consisted of 0.1 ml of undiluted stock virus ($ID_{50}$ $10^{-3.5}$). Following 30 minute adsorption period on roller drum, 0.9 ml of medium A was added to each tube. Duplicate tubes were removed at this time (0 hour) and 3-5 tubes were removed at intervals (48 or 96 hours) thereafter. The contents of each series were pooled, ground in Ten Broeck grinder, ampulated, and stored in a dry-ice chest prior to assay in newborn mice. Subsequently, 10 serial passages were made. For calculation of $ID_{50}$ endpoints, the original inoculum of 10% liver homogenate (stock virus) was considered as undiluted. Except for serial passages 2 and 5, successive cultures were inoculated with 0.1 ml of their preceding cultures. The 2 exceptions were inoculated with 0.1 ml of 1:10 dilutions (made in PBS) of their respective preceding cultures. Theoretical $ID_{50}$ endpoints at 0 hour are included in Table I for comparison with the 0 hour samples. For successive cultures, these values were 0.1 of the calculated $ID_{50}$ endpoints of their preceding cultures in all cases except serial passages 2 and 5. For the latter cases, theoretical values were 0.01 of their respective preceding cultures. The $ID_{50}$ endpoints of successively passaged cultures ranged from $10^{-4.1}$ to $10^{-5.2}$ with average of $10^{-4.6}$ (Table I). The theoretical average at 0 hour was $10^{-3.3}$ and varied slightly from the calculated 0 hour average $10^{-3.0}$. In several instances, theoretical titres were slightly higher or equal to the calculated 0 hour titres, possibly indicating decay or adsorption of virus by the proliferating explants. However, for passages 2 and 8, the reverse situation occurred and may be within the range of experimental error. Later experiments indicated that adsorption had occurred and that extracellular virus was infective for newborn mice.

Total time of virus growth (636 hours) during the 10 passages in tissue culture far exceeded virus decay rate at 37°C in medium A and indicated that virus propagation had occurred. In addition, virus inoculum was diluted to $10^{-12}$ of its original concentration.

TABLE II. Comparison of Infectivity of Successive Culture Fluids with Whole Kidney Explant Cultures.

| Series | Culture fluids | | Whole cultures |
|---|---|---|---|
| | Hr | $-\log ID_{50}$ | $-\log ID_{50}$ |
| | | | Hr |
| a | 0 | 2.6* | 2.7 ( 0) |
| | 120 | 2.8 | |
| b | 72 | 4.5 | 4.5 (72) |
| | " | 3.8 | |
| | 96 | 3.3 | |
| c | 96 | 4.3 | 4.3 (96) |
| | 72 | 3.3 | |
| | " | 3.9 | |

* Theoretical $ID_{50}$ of intracellular virus which remained after removal of 0 hr culture fluids was $10^{-0.6}$.

Cytopathogenicity was noted in several instances but such effects occurred erratically. Occasionally, cells became enlarged, refractile, and grape-like clusters were formed as has been described for canine infectious hepatitis(9). Cell destruction usually occurred around the periphery of explant growth. These observations are being studied in greater detail.

*Infectivity of tissue culture fluids.* Kidney explants were grown in medium A for 48 hours at which time confluent sheets of cells had formed. Media were removed and explant cultures were inoculated with 0.1 ml of serial passaged tissue culture 9 ($ID_{50}$ $10^{-4.2}$). After 30 minute adsorption period on roller drum, 0.9 ml of medium A was added to each tube (theoretical $ID_{50}$ $10^{-3.2}$). Nutrient fluids were removed for assay and replaced with 1 ml of medium A at time intervals (Table II). Three series of cultures (a, b, and c) were prepared. It should be noted that 0 hour cultures (series a) were rinsed twice with 1 ml of PBS following adsorption period. For comparison with the titres of culture fluids, whole cultures were assayed after 0, 72, and 96 hours.

The data shown in Table II suggest that 1) virus is adsorbed by proliferating explant cultures, 2) virus propagation had occurred, and 3) only extracellular virus may be infective. Adsorption of virus is shown by decrease in titre at 0 hour (series a) from a theoretical $ID_{50}$ endpoint $10^{-3.2}$ to $10^{-2.6}$. The difference ($10^{-0.6}$) represents the potential $ID_{50}$ adsorbed by kidney explants and which remained after rinsing the inoculated explants with PBS. Further evidence that adsorption and propagation had occurred is shown by subsequent rise in titre ($ID_{50}$ $10^{-2.8}$) after 120 hours. In series b and c, the final dilution of the original inoculum after 3 successive changes of nutrient medium exceeded the $ID_{50}$ endpoint of the inoculum and total time of these experiments (240 hours) exceeded decay time of the virus. The similarity of titres obtained with entire cultures and with culture fluids alone at comparable time intervals (0, 72, and 96 hours) suggests that only the culture fluids contain the mature virus.

*Summary.* The Gledhill strain of mouse hepatitis virus (MHV 1) was passaged through 10 successive tissue cultures of newborn mouse kidney explants. As MHV 1 did not cause reproducible cytopathogenic effects, infectivity tests with newborn mice were used to detect propagation of this virus in serially passaged cultures. Observations suggest that virus was adsorbed by the proliferating explants and that cell-free culture fluids were infective. Total time of virus growth far exceeded decay rate of the virus incubated without tissue. After the tenth passage, the virus had been diluted $10^{12}$-fold which exceeded the extinction point of the original inoculum.

1. Gledhill, A. W., *J. Gen. Microbiol.*, 1956, v15, 292.

2. Dick, G. W. A., Niven, J. S. F., Gledhill, A. W., *Brit. J. Exp. Path.*, 1956, v37, 90.

3. Levine, P. P., Fabricant, J., *Cornell Vet.*, 1950, v40, 71.

4. Asplin, F. D., *Vet. Rec.*, 1956, v68, 412.

5. Hanson, L. E., Alberts, J. O., *J. Am. Vet. Med. Assn.*, 1956, v128, 37.

6. Rubarth, S., *Acta Path. Microbiol. Scand. Suppl.*, 1947, v69, 1.

7. Havens, W. P., Jr., PROC. SOC. EXP. BIOL. AND MED., 1945, v58, 203.

8. Buescher, E. L., *Ann. Historical Rep. of 406 Gen. Med. Lab., U. S. Army,* 1952, (cited in ref. 12)

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

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

11. Emery, J. B., York, C. J., *Science,* 1958, v127, 148.

12. Miyazaki, Y., Katsuta, H., Aoyama, Y., Kawai, K., Takaoka, T., *Japan, J. Exp. Med.,* 1957, v27, 381.

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

14. Earle, W. R., Nettleship, A., Schilling, E. L., Stark, T. H., Straus, N. R., Brown, M. F., Shelton, E., *J. Nat. Cancer Inst.,* 1943, v4, 165.

15. Fernandes, M. V., *Texas Rep. Biol. Med.,* 1958, v16, 48.

16. Gey, G. O., Coffman, W. D., Kubicek, M. T., *Cancer Research,* 1952, v12, 48.

17. Pollard, M., *Ann. N. Y. Acad. Sci.,* 1958, v70, 383.

18. Nelson, J. B., *J. Exp. Med.,* 1952, v96, 293.

Received October 10, 1958. P.S.E.B.M., 1959, v100.

## Formation of a Pharmacologically Active Substance from Plasma Protein.* (24537)

C. G. HUGGINS AND E. J. WALASZEK† (Introduced by G. L. Curran)

*Dept. of Pharmacology, Kansas University School of Medicine, Kansas City, Kan.*

A number of investigators(1,2) have shown that vasodilator polypeptide, bradykinin, can be produced by incubating plasma protein with crystalline trypsin. Also that an enzyme capable of producing bradykinin upon incubation with plasma protein can be released from salivary gland cells by stimulation of the chorda tympani(3) and that bradykinin so produced has a role in regulation of blood flow in the submandibular salivary gland. It occurred to us that the enzyme $\alpha$-amylase might be important in production of bradykinin, since salivary glands are rich in $\alpha$-amylase. Furthermore, fraction IV-4 of plasma protein which contains bradykininogen, the precursor of bradykinin, is rich in glycoprotein which could well be susceptible to hydrolysis by $\alpha$-amylase. Therefore it seemed conceivable that the action of $\alpha$-amylase on glycoprotein might lead to production or activation of bradykinin. Saliva incubated with blood produced vasodilator principle bradykinin(3) and $\alpha$-amylase is present in saliva along with small amounts of other enzymes.

*Materials and methods.* Fraction IV-4 of plasma protein was obtained through joint cooperation of American Red Cross and E. R. Squibb. Enzymes and enzyme inhibitors were obtained from commercial sources except salivary amylase, which was prepared from pooled saliva and partially purified by the method of Bernfeld(4). Detection and extent of formation of the pharmacologically active material in the incubation mixture was measured by ability to contract isolated segments of guinea-pig ileum suspended in 10 ml organ bath and attached to frontal writing lever for recording on smoked drum of kymograph. The bath fluid was Tyrodes' solution at 37°C. The effect of pharmacologically active polypeptides on blood pressure was studied in dogs and rabbits anesthetized by intraperitoneal injection of sodium pentobarbital (30 mg/kg) and urethane (1-1.5 g/kg), respectively. Pressure was recorded from cannulated carotid artery with a mercury manometer. All injections were made *via* cannulated femoral vein. Formation of pharmacologically active material was carried out by incubating equal volumes of 0.5% $\alpha$-amylase and 0.5% fraction IV-4, both dissolved in Tyrodes solution at 37°C. At appropriate times aliquots were removed and tested for activity on the guinea pig ileum. The active material was in contact with the tissue for 60 seconds. Aliquots of the incubation mixture, removed at appropriate times, were also injected I.V. into anesthetized dogs and rabbits for measurement of effects on blood pressure. Formation of

* Presented before Am. Soc. for Pharmacol. and Exp. Therap., Ann Arbor, Mich., Aug. 1958.

† This investigation was supported by Senior Research Fellowship SF-72 from P.H.S.