located in the supratrigeminal nuclear region; (b) induced a phase of excitation that was partially obscured by the early inhibition; (c) induced a long latency inhibition via a polysynaptic path which extended caudal to the motor nucleus of V[11].

Zusammenfassung. Elektrische Reizung afferenter Fasern niedriger Reizschwelle im Nervus lingualis induzierte in den motorischen Neuronen des Nervus massetericus: (1) IPSPs von kurzer Latenz mittels einer bisynaptischen Bahn, deren Zwischenneurone im Gebiet der supratrigeminalen Nuclei liegen; (2) eine Erregungsphase, die teilweise von einer früh auftretenden Hemmung verdeckt ist; (3) eine Hemmung von langer Latenz mittels einer polysynaptischen Bahn, die sich kaudalwärts vom motorischen Ursprungskern des Nervus trigeminus erstreckt.

L. J. Goldberg and Y. Nakamura

Department of Anatomy and the Brain Research Institute, University of California, Los Angeles (California 90024, USA), 6 November 1967.

[11] This research was supported by a grant from the USPHS (No. MH-10083). Dr. Goldberg is a NIDR Postdoctoral Fellow. Dr. Nakamura is a Research Fellow from the Department of Neurophysiology, Institute of Brain Research, University of Tokyo (Japan).

Propagation of Mouse Hepatitis Virus (MHV-3) in Monolayer Cell Cultures from Liver of New-Born Mice

Mouse hepatitis virus (MHV-3) has been propagated in vitro with different techniques by several authors[1-4] and, particularly, in the cells derived from liver explants of mice fetuses and cultivated on reconstituted rat-tail collagen[5]. With this technique the virus replication was followed by an evident cytopathic effect.

This method is, nevertheless, very complicated and the cell growth is very slow. On the other hand, it is known that the liver cells obtained from fetuses or from new-born animals do not survive long when cultivated on a plain glass surface.

In order to gather further information on MHV-3 replication in vitro, it seemed of interest to test the possibility of replication of this virus on primary monolayer liver cell cultures obtained from new-born mice with a simple technique.

Materials and methods. Cell culture: The livers of new-born mice (Swiss strain) were pooled, finely minced with scissors, twice washed in cold Hanks' BSS, placed in 0.25% trypsin (Difco) and 0.20% methylcellulose (Fischer) in calcium- and magnesium-free Hanks' BSS, and agitated on a magnetic stirrer for 20–30 min at room temperature. The resulting suspension was centrifuged at 300 *g* for 10 min, decanted, and the sediment resuspended in the nutrient medium. The nutrient medium consisted of 20% of fresh inactivated calf serum, 0,5% of chick embryo extract and 0.1% of yeast extract (Difco) in Eagle's basal medium (Difco). The cells were counted, diluted in the nutrient medium to a concentration of 1×10^6/ml, distributed in 2 ml volumes in stationary tubes or in Leighton tubes containing a coverslip for the morphological observations, and placed in a 37 °C incubator. After 7–10 days a monolayer of polygon-shaped cells was formed. Subcultures were performed approximately every 10 days, using the same method described above.

These cells were passed through 9 subcultures; after this time degeneration of the cultures occurred.

Virus: MHV-3 was supplied by the American Type Culture Collection and maintained in our laboratory in receptive albino mice. The LD_{50} was determined according to Reed and Muench by using 3-week-old albino mice (Swiss strain) weighing about 10 g.

A 10^{-3} dilution of liver homogenates from moribund animals in Eagle's basal medium was made extemporaneously and centrifuged at 3000 rpm for 10 min.

For culture infection, the nutrient medium was eliminated and 2 ml of fresh nutrient medium containing 0.1 ml of viral suspension were placed in the tubes. The cultures were maintained at room temperature for 60 min. The virus suspension was then eliminated, the cell cultures twice washed with Hanks' BSS and incubated at 37 °C with fresh growth medium.

After 0, 12, 24, 36, 48 and 72 h respectively, the culture medium from 10 infected tubes was pooled and 10-fold diluted in sterile broth.

The biological virus titration was carried out with 0.1 ml of each dilution inoculated i.p. into 10 albino mice. At the same time, the coverslips were extracted from the

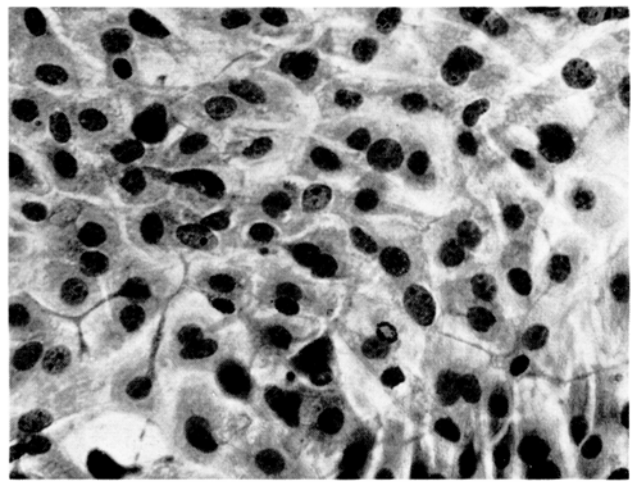

Fig. 1. Epithelial cell culture from trypsinized liver of new-born mice (8 days after trypsinization). Hematoxylin-eosin. × 250.

[1] R. F. Haff, Virology *18*, 507 (1962).
[2] T. Vainio and J. D. Judah, Expl molec. Path. *1*, 27 (1962).
[3] J. W. Hartley and W. P. Rowe, Proc. Soc. exp. Biol. Med. *113*, 403 (1963).
[4] L. Mallucci, Virology *25*, 30 (1965).
[5] F. Paradisi and F. Piccinino, G. Microbiol., in press.

Leighton tubes and the specimens were fixed and stained with hematoxylin-eosin for the morphological observations.

Some cell cultures, as control, were treated as infected cultures, but a liver suspension from healthy animals was substituted for virus suspension.

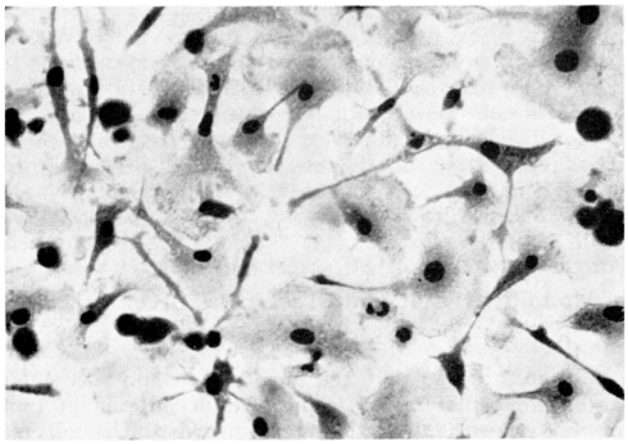

Fig. 2. Cytopathic effect induced by MHV-3 in cell culture of liver from new-born mice (36 h after virus infection). Hematoxylin-eosin. × 250.

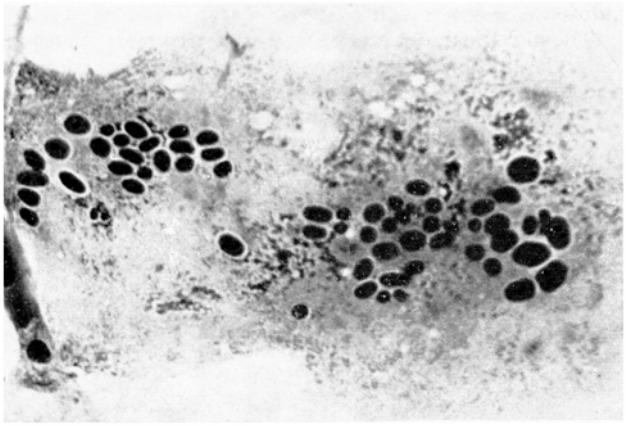

Fig. 3. Syncytial formations in culture of liver cells from new-born mice 48 h after infection with MHV-3. Hematoxylin-eosin. × 400.

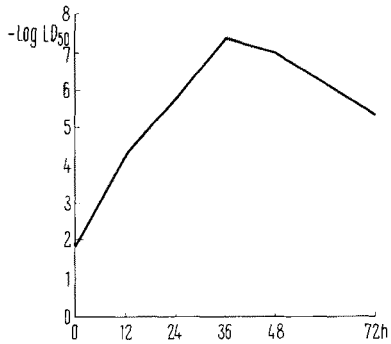

Fig. 4. Growth of MHV-3 in cultures of liver cells from new-born mice.

Observations. MHV-3 has been replicated actively in this cell culture (see Figure 4) and a cytopathic effect occurred within about 24 h. Hematoxylin-eosin staining showed various stages of nuclear pycnosis and cytolysis, the cells tended to form clumps and become detached (Figure 2). After 36 h large syncytial formations containing 10–30 pycnotic nuclei appeared (Figure 3).

The virus passages from culture to culture showed the same picture. The increase and decrease of virus concentration were rather rapid, and the maximum titre was at 36 h.

With the histological technique, we were unable to demonstrate any cytoplasmic inclusion as observed by some authors[5,6] in different cell cultures infected with MHV-3. Normal liver suspension did not produce any detectable effect on these cell cultures.

Comment. The cultivation of MHV-3 in monolayer of new-born mice liver cell cultures is a very simple method which gives constant results. MHV-3 replicates actively in these cultures and high virus concentration is achieved in the culture medium.

It is an easier and simpler method than the cultivation in cell cultures derived from liver explants on reconstituted rat-tail collagen because a quick and constant cell growth can be obtained. In optimal conditions the liver cells grew into a complete sheet and fibroblastic cells could only occasionally be recovered.

Liver cell cultures are ordinarily obtained from liver explants, because numerous laboratories have experienced difficulties in trypsinized monolayer liver cell cultures.

The addition of methylcellulose to trypsin solution greatly increases the number of viable cells. It supposedly acts by protecting the cell surface and by influencing the aggregation of cells[7]. The presence of this substance in trypsin solution is essential to obtain long-term chicken embryo heart cell cultures[8].

The chicken embryo extract, at the concentration of 0.5% in the nutrient medium, appears to be indispensable for cell growth. Eagle's basal medium without chicken embryo extract produces an inadequate cell growth with a prevalence of fibroblastic cells. Chicken embryo extract concentrations higher than 0.5–1.0% are unable to enhance ulteriorly the cell growth[9].

Riassunto. È stata provata la possibilità di coltivare MHV-3 in colture monostrato di cellule di fegato di topo neonato ottenute per tripsinizzazione. Per questo tipo di coltura è indispensabile l'aggiunta alla tripsina di metilcellulosa e di estratto embrionale di pollo al terreno di coltura. MHV-3 si moltiplica attivamente in queste colture, raggiungendo elevate concentrazioni nel liquido di coltura e producendo effetto citopatico.

F. PARADISI and F. PICCININO

Clinica delle Malattie Infettive della Università di Napoli, Osp. Gesù e Maria, Napoli (Italy), 2 October 1967.

[6] T. VAINIO, Proc. Soc. exp. Biol. Med. *107*, 326 (1961).
[7] D. J. MERCHANT and K. B. HILLMAN, Proc. Soc. exp. Biol. Med. *110*, 194 (1962).
[8] H. SCHNEIDER, M. W. SHAW, E. E. MUIRHEAD and A. SMITH, Expl Cell Res. *39*, 631 (1965).
[9] Acknowledgment: The skilled technical assistance of Mr. R. GENTILE is gratefully acknowledged. – This work was supported by the Italian Consiglio nazionale delle Ricerche, Impresa di Virologia.