Growth of Mengo Encephalitis Virus in Ehrlich Ascites Cells in Vitro

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Since the Ehrlich ascites tumor cells were first described by Loewenthal and Jahn (14), they have been the subject of many investigations, both biological and chemical. Klein investigated the biology and nucleic acid content of the cells (8, 9). Their striking property of assimilating and concentrating free amino acids was reported by Christensen and co-workers (2). Kun and others studied some of the enzymatic and metabolic activities of the ascites cells (11), and McKee and his associates (15) presented findings on the endogenous respiration and the effect of some of the Krebs cycle intermediates upon the respiration of these cells. Koprowska and Koprowski (10) were successful in growing Mengo encephalitis virus in the Ehrlich cells in vitro, and Love, Koprowski, and Cox (13) described cytological changes associated with the growth of Bunyamwera virus in Ehrlich ascites tumor cells in mice. The successful propagation of ascites tumor cells in tissue culture (7, 12, 19) suggested the possibility of investigating the virus infection of the cells in vitro.

This paper reports the propagation of Mengo encephalitis virus in Ehrlich ascites cells by simple tissue culture technic and preliminary studies on some of the metabolic activities of the uninfected and virus-infected tumor cell systems.

MATERIALS AND METHODS

Virus.—The Mengo virus, a member of the encephalomyocarditis group of viruses, was originally isolated in Uganda, Africa (4). The strain used in the present studies was made available to Dr. Hilary Koprowski of this laboratory through the kindness of Dr. George Klein of the Karolinska Institute in Stockholm. An original inoculum of 10 million cancer cells was given to mice. On the 7th–9th day of tumor growth, ascitic fluid was obtained by peritoneal tapping of from four to ten mice. A 10-ml. syringe equipped with a 20-gauge needle, both previously rinsed with heparin (USP), was used, and the fluid was transferred to a chilled 50-ml. centrifuge tube containing 0.20 ml. of heparin. The ascitic fluid was centrifuged in an International angle-head refrigerated centrifuge, Model PR-1, at 2,500 r.p.m. for 10 minutes at 4° C. The ascitic plasma was decanted, and the cells were transferred with a glass spatula, care being taken to avoid the transfer of sedimented erythrocytes, to another 50-ml. centrifuge tube containing approximately 55 ml. of chilled, phosphate-buffered physiological saline solution (PBS) (5). After being vigorously shaken by hand, the cells were re-centrifuged at 2,500 r.p.m. for 10 minutes. The saline washing was repeated 2 or 3 times to remove all red blood cells.

Virus-cell suspensions.—Washed ascites cells were resuspended in an arbitrary volume of Parker's synthetic medium 199 (17), and the number of cells was counted in a standard bright-line Neubauer hemocytometer. The average of four counts was taken to determine the cell concentration. Penicillin and streptomycin were routinely used in all cultures to give final concentrations of 200 units and 200 μg., respectively. Virus was added in the concentrations noted in each experiment.

Two-ml. aliquots of the cell-virus suspensions were transferred to 15 × 150 mm. Pyrex test tubes, closed with gum rubber stoppers, placed in a roller drum, and incubated at 37° C. for 24–120 hours. After the desired interval of incubation, the cultures were harvested, pooled, and stored in rubber-capped vaccine vials at -20° C. until the virus titrations in mice were performed.

Bioassays.—Bioassays of Ehrlich ascites cell tissue cultures were carried out after various intervals of incubation by the subcutaneous injection into 15–20 gm. Swiss albino mice of 0.3–ml. of undiluted pooled tissue culture supernatant fluid containing those cells which did not adhere firmly to the test
tube walls. At least six mice were given injections in each assay. These mice were examined daily for 7 days for the presence of progressively growing tumors at the site of injection.

Biochemical studies.—
Oxygen uptake: Two-ml. aliquots of cell suspensions were placed in standard single or double side-arm Warburg vessels, and 0.2 ml. of 20 per cent KOH was placed in the center wells. The vessels were attached to standard manometers and placed in a 37° C. water bath. The oxygen uptake was measured at 30-minute intervals for periods of 1-2 hours. The gas phase in all the experiments was air.

Glucose determination: Half of the pooled aliquots taken from the Warburg vessels after oxygen uptake had been measured was used for glucose determination. The material was deproteinized with Ba(OH)2 and ZnSO4, and estimations were performed on the protein-free filtrates according to Nelson's modification of Somorgyi's method (16).

Lactate estimations: The portion of the pooled aliquots not used for glucose determination was deproteinized with 20 per cent trichloroacetic acid and, after standing for 60 minutes at 4° C., was filtered through Whatman #1 filter paper. Lactate was determined on aliquots of the neutralized filtrate according to the method of Barker and Summerson (1).

RESULTS
Choice of culture medium.—Oxygen uptake studies were undertaken of Ehrlich ascites cells in the following media, and in phosphate-buffered saline (PBS) as a reference, to find which showed the best capacity for sustaining the viability of the cells: (a) Parker's 199; (b) 50 per cent Parker's 199, 40 per cent normal horse serum, and 10 per cent chick embryo extract. The results, shown in Table 1, indicate that oxygen uptake in Parker's 199 was significantly lower than that in the complex medium or in the PBS.

To determine in which of the media the virus would best propagate, aliquots of cells suspended in Parker's 199 and in the complex medium were then infected with one mouse LD50 of Mengo virus per 1 million cells and incubated for 48 hours. A titer of $10^{-4}$ was obtained in mice for infected cells which had been grown in 199 alone. However, no virus could be detected when mice received inocula which had been incubated with horse serum. Several neutralization tests were performed to ascertain whether normal horse serum inhibited the multiplication of Mengo virus, and the results showed that horse serum had a neutralizing index of 1.17 logs, or the ability to neutralize approximately 147 mouse LD50—more virus than was used as inoculum. Therefore, since the medium containing horse serum had proved to be unsuitable for the growth of Mengo virus, even though a better medium for sustaining the viability of the Ehrlich cells, Parker's 199 alone was used in the remainder of the experiments.

Effect of incubation on viability of Ehrlich cells.—Cell suspensions of 5 million/ml were prepared in medium 199, and tubes containing 2-ml. aliquots were incubated for 48 and 96 hours at 37° C., after which procedure the contents were bioassayed. All the mice given injections of the suspension which had been incubated for 48 hours developed tumors, whereas tumors grew in 20-80 per cent of those receiving the inoculum which had been incubated for 96 hours.

Effect of inocula and time of incubation on virus yields.—Experiments were undertaken to find what concentration of virus inoculum and what time of harvest produced optimum virus yields. Concentrations of Mengo virus from $5 \times 10^2$ to 0.5 mouse LD50 were added to Ehrlich cell cultures containing $5 \times 10^4$ cells/ml. The cultures were incubated for 48 hours, then harvested, pooled, and titrated intracerebrally in mice. Chart 1 shows the results. The optimum virus inoculum was apparently 5 mouse LD50, since the yields of virus in excess of the inocula decreased when more concentrated inocula were employed.

The most favorable time to harvest was next investigated. Cultures containing 5 million/ml Ehrlich cells and 5 mouse LD50 of virus were harvested after 24, 48, 72, 96, and 120 hours, and their virus content was determined by mouse titration. Maximum virus yields were obtained at 48 hours, with a subsequent leveling off in an-
other 24 hours, followed by a drop thereafter (Chart 2).

Five million ascites cells to 5 mouse LD_{50} of Mengo virus was shown to be a satisfactory ratio in fifteen experiments in which this proportion of cells to virus resulted in cultures giving, upon titration in mice, titers of 10^{-4.4} to 10^{-7.4}, with an average of 10^{-5.4} and a corresponding average log increase in virus content of 5.8.

Metabolic studies.—The rates of oxygen uptake, glucose utilization, and lactate production in Mengo-infected Ehrlich cell cultures and in uninfected control cultures were studied. First, these activities were measured over an initial 2-hour incubation period. Second, the metabolic determinations were made following incubation for various intervals and resuspension of the cells in fresh medium. In each experiment a dilution of Mengo-infected mouse brain pool giving a ratio of 5,000 mouse LD_{50} per 1 million cells was used.

As seen from Table 2, the total oxygen used over a 2-hour period of incubation at 37°C was relatively constant in both infected and uninfected systems. The rate of glucose utilization was high, with nearly complete disappearance of glucose from the medium after 2 hours. Lactate accumulation did not account for this rapid disappearance of glucose. There was no significant difference in the rates of oxygen consumption, glucose utilization, or lactate production between the infected and uninfected cells.

In the next experiments, tissue cultures were prepared in silicone-coated flasks and incubated at 37°C for 8, 12, and 18 hours. During the time of incubation the flasks were gently shaken in a horizontal shaker patterned after that described by Daniels and others (3). After incubation, the contents of five culture flasks were transferred to a 50-ml centrifuge tube and centrifuged at 2,500 r.p.m. for 10 minutes, washed once with PBS, re-centrifuged, and resuspended in a volume of fresh medium 199 to give a concentration of 5 × 10^{6} cells/ml. Oxygen uptake and glucose utilization of these resuspended cells were measured as described previously (Table 3). As can be seen by a comparison with Table 2, no significant changes in oxygen consumption occurred in cells cultured for various periods. Essentially no evidence of glucose utilization could be detected after 1 hour of incubation in fresh medium.

Virus passage.—The serial passage of Mengo encephalitis virus in Ehrlich ascites cells in vitro was begun with a ratio of 1 mouse LD_{50} per 1 million cells. Tubes were incubated for 48 hours at 37°C, after which the contents were harvested and pooled. Material from this pool in a final dilution of 10^{-4} served as inoculum for the second pas-

<table>
<thead>
<tr>
<th>Duration of Incubation (hours)</th>
<th>O_2 Uptake/10^6 Cells</th>
<th>Glucose Utilization/10^6 Cells</th>
<th>Lactate Produced/10^6 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uninfected</td>
<td>Mengo-infected</td>
<td>Uninfected</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>12</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>32.2</td>
<td>37.2</td>
<td>35.5</td>
</tr>
</tbody>
</table>

* Figures represent total O_2 uptake in 60 minutes.
shown in Table 4, indicate that the agent passed can be identified as Mango encephalitis virus.

Effect of trypsin on ascites cells infected with Mango virus.—There were no observable cytopathogenic effects, under the experimental conditions described, upon the ascites tumor cells after incubation with Mango encephalitis virus; a large number of cells adhered to the culture tube walls and could not be removed by vigorous shaking or scraping with a glass spatula. When a 0.25 per cent trypsin solution (Difco, 1:250) in PBS was added to culture tubes infected with serial dilutions of Mango-infected mouse brain and to uninfected controls, the cells were removed from the walls. In the infected cultures, a rather dense white coagulum appeared which left the supernatant fluid clear. This coagulum could be redispersed either before or after centrifugation to remove the trypsin solution. The centrifuged cells were resuspended in an amount of PBS equivalent to the volume present before trypsin was added. Occasionally tiny, mucin-like threads were seen, but the supernatant fluid had the characteristic turbidity of a cell suspension.

To ascertain whether the apparent cell destruction after trypsinization was considerable when the original virus inoculum was large, but when the inoculum was 50 mouse LD50 or less, some visibly unaltered cells remained. As the virus inoculum was decreased, the numbers of these intact cells increased markedly.

To ascertain whether the apparent cell destruction was caused by Mango virus, serial tenfold dilutions of Mango-infected mouse brain were mixed with equal quantities of (a) Mango immune monkey serum, (b) normal monkey serum, and (c) medium 199, and were incubated at 37°C for 30 minutes. After incubation the virus dilutions were inoculated into Ehrlich cell cultures. Forty-eight hours later, 4.0 ml. of trypsin solution was added to all tubes. Ten minutes after the addition of trypsin, the tube contents were removed and pooled in 50-ml centrifuge tubes. The coagulum was redispersed, and the tubes were centrifuged for 10 minutes at 1,000 r.p.m. The trypsin solution was removed by suction; the sediments were resuspended in PBS; and the cells were counted. The results (Table 6) showed the cell counts in the cultures containing virus plus normal serum and virus alone to be significantly decreased. Also, no appreciable differences were noted in the cell counts between the virus-Mango immune serum cultures and the uninfected controls. As might be expected, virus titers were obtained only with material from infected cultures to which no anti-Mango serum had been added.

**TABLE 4**

<table>
<thead>
<tr>
<th>Virus Source</th>
<th>Titer in mice when mixed with</th>
<th>Titer in mice when mixed with</th>
<th>Neutralizing index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sixth tissue culture passage</td>
<td>Normal monkey serum</td>
<td>10^{-2.2}</td>
<td>10^{-1.9}</td>
</tr>
<tr>
<td>Mango-infected mouse brain pool</td>
<td>Mango immune monkey serum</td>
<td>10^{-1.1}</td>
<td>10^{-0.8}</td>
</tr>
</tbody>
</table>

**DISCUSSION**

These studies indicate that Ehrlich ascites cells can, under the proper experimental conditions, support the growth of Mango encephalitis virus in vitro. Virus was recovered in quantities larger than were inoculated into the cell cultures; the virus was propagated for eleven successive passages, representing a dilution of the original virus-infected mouse brain pool of 10^{-44}; and the titer of the eleventh in vitro passage was 10^{-7.0}. Moreover, specific anti-Mango monkey serum prevented the growth of Mango virus when mixed with the virus suspension before inoculation into the cultures, while normal monkey serum exerted little, if any, effect on the virus titer (Table 6). Virus from the sixth and eleventh tissue culture passages was
identified by neutralization tests as Mengo encephalitis virus (Table 4).

The maximum increase of Mengo virus from Ehrlich cell cultures containing $5 \times 10^4$ cells/ml was between 6 and 7 logs. This occurred when a ratio of approximately 1 mouse LD$_{50}$ to 1 million cells was employed. When more concentrated inocula were used the log increase of virus decreased (Table 5, Chart 1). Thus, when 50,000 mouse LD$_{50}$ of Mengo virus were inoculated into Ehrlich cell cultures, the average log increase of virus, in excess of the inoculum, was only 2.8. This may have been caused by interference or, more probably, since no evidence of cellular proliferation of Ehrlich cells in medium 199 could be demonstrated, by the limited cell population available for viral growth.

### SUMMARY

A simple tissue culture method has been described by which Mengo encephalitis virus can be propagated in Ehrlich ascites cells. The carcinoma cells can be maintained for a period of at least 48 hours in a completely defined synthetic medium. The greatest yield of virus was obtained when a ratio of 1 million Ehrlich ascites cells to 1 mouse LD$_{50}$ of Mengo encephalitis virus was employed. Preliminary results of some metabolic studies of normal and Mengo-infected ascites cell cultures are reported.

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