Replication of Variola Virus in Suspended Cultures of Mammalian Cells

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ABSTRACT

MIKA, LEONARD A. (U. S. Army Chemical Corps, Frederick, Md.), and JAMES B. PIRSCH. Replication of variola virus in suspended cultures of mammalian cells. Appl. Microbiol. 9:545–548. 1961.—The studies reported here describe the successful propagation of variola virus in spinner cultures of mammalian cells, and the factors which influence its growth. Five established cell lines were used for the propagation of variola virus in a spinner culture system. Low doses of virus did initiate an infection but virus yields did not approach those obtained when an intermediate inoculum was used. Although the nonviable cell population remained low during the course of infection with an intermediate amount of virus, with an inoculum of 10⁴ infectious units per ml or higher, the percentage of nonviable cells increased rapidly and by the sixth day after infection the population was totally nonviable. Intracellular replication of variola virus occurred early and rapidly in a spinner culture of guinea pig lung cells, whereas the liberation of virus into the suspending medium was a more gradual process. Several complete medium changes tend to maintain a suitable environment for the infected cell culture resulting in fairly high and constant viral titers over a period of 7 days.

The propagation of variola virus in monolayer cultures of various tissue cells has been investigated by Boué and Baltazard (1956), Baltazard, Boué, and Siadat (1958), Hahon (1958), Vieuxchane, deBrion, and Gruest (1958), Hahon and Kozikowski (1959), Maren-nikova, Gurvich, and Yemansheva (1959), and Pirsch and Mika (1960). Apparently no study has been made on the ability of variola virus to grow in a suspended cell system. The work reported here describes the successful propagation of variola virus in spinner cultures of mammalian cells, and it also concerns some factors which influence its growth.

MATERIALS AND METHODS

Virus seed. The Yaman strain of variola virus, characterized by Hahon, Ratner, and Kozikowski (1958), was used throughout these studies. A 10% suspension in heart infusion broth (Difco)¹ was pre-

¹ Difco Laboratories, Inc., Detroit, Mich.
TABLE 1. Growth of variola virus in submerged (spinner) cell cultures

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Reference</th>
<th>Infective dose</th>
<th>Peak titer*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guinea pig lung</td>
<td>Isolated in our laboratories</td>
<td>3.7 X 10⁴</td>
<td>6.2 X 10⁷</td>
</tr>
<tr>
<td>Maben (CA of Human Lung)</td>
<td>Friech et al. (1955)</td>
<td>1.0 X 10⁴</td>
<td>4.1 X 10⁷</td>
</tr>
<tr>
<td>Chang human liver</td>
<td>Chang (1954)</td>
<td>9.0 X 10⁴</td>
<td>1.9 X 10⁷</td>
</tr>
<tr>
<td>Embryonic human lung</td>
<td>Henle and Deinhardt (1957)</td>
<td>6.8 X 10⁴</td>
<td>1.8 X 10⁷</td>
</tr>
<tr>
<td>L (altered mouse fibroblast)</td>
<td>Sanford, Earle, and Likely (1948)</td>
<td>9.0 X 10⁴</td>
<td>9.5 X 10⁶</td>
</tr>
</tbody>
</table>

* Attained in 4 to 5 days.

Fig. 1. Growth of variola virus in spinner culture of guinea pig lung cells.

The growth pattern of variola virus in a spinner culture of guinea pig lung cells is shown in Fig. 1. A slight rise in virus titer with no apparent early lag was observed within the first 2 days. A plateau maintained for the next day was followed by a rapid increase until peak titer was reached on the fifth day. The percentage of nonviable cells remained low during this entire period; this pattern paralleled that obtained in the uninfected cell-culture control. Nonviable cells in both infected and uninfected cultures varied usually from 5 to 20%.

**Effect of viral inocula.** In preliminary experiments low viral inocula, of the order of 10⁴ to 10⁵ IU per ml, were used to determine whether small amounts of virus would yield appreciable titters in a spinner culture system. The results showed that a 10- to 100-fold increase in virus titer was obtained with these inocula after prolonged cultivation. In comparison, when a viral inoculum of over 10⁶ IU per ml was employed, a maximal titer of 1 × 10⁷ IU per ml was reached on the third day after infection (Table 2). Under these latter conditions, the number of nonviable cells in the infected culture increased dramatically until 50% of the cell population was nonviable. By the sixth day, this cell population was totally nonviable. No such increase in the nonviable population of the uninfected control culture was detected.

**Growth kinetics of the virus.** The data showing the pattern of virus replication in the various components of an infected spinner culture of guinea pig lung cells...
are presented in Fig. 2. The viral titer of the supernatant fluid decreased tenfold within the first 12 hr. By 24 hr it reached the initial (0 hr) titer, followed by a steady increase until a maximal titer of $1.0 \times 10^7$ IU per ml was reached at 96 hr. Thereafter, a slow decline was noted to the termination of the experiment. In contrast, little or no increase in viral titer was observed in the disrupted cell milieu during the first 6 hr. However, from 6 to 24 hr postinfection, an 850-fold increase of intracellular virus was detected. A maximal titer of $9.5 \times 10^4$ IU per ml was obtained by 72 hr and this was followed by a gradual although consistent decline up to 168 hr. This pattern coincides with a loss in viability of the infected cell population, that is, by 72 hr, 60% of the cells were considered nonviable.

**Effect of medium changes.** Studies were made to determine what effect frequent medium changes would have on a spinner culture of guinea pig lung cells infected with an intermediate dose of variola virus. Complete changes (100 ml) were made 2, 4, and 6 days after infection. In addition to the usual daily samples of the intact culture, the following aliquots were prepared when medium changes were made: infected cells (after centrifugation and resuspension in fresh medium) and supernatant fluids after centrifugation (absence of tissue cells). There appeared to be no discernible differences in titers between intact cultures and the infected cells resuspended in fresh medium after 2 days (Table 3). However, at the same time, supernatant fluid contained about 75-fold less virus than the resuspended cells. After 4 days the titers were nearly equal but by the sixth day some increase in extracellular virus was noted (5.5-fold).

**DISCUSSION**

The propagation of variola virus in spinner cultures of mammalian cells is feasible, and some subtle effects of the virus on the suspended cells have been noted. The virus yield in spinner culture has approached the titers obtained in monolayer systems especially when an intermediate dose of virus was chosen as the inoculum. Under these conditions, the viable cell population appeared to be only slightly altered during the course of the infection.

Although small amounts of virus do initiate an infection in the suspended cell system, the titers achieved were never as high as those obtained in a monolayer cell culture similarly infected. The lack of greater virus multiplication may be attributed to the mechanics of the infection in spinner culture since maximal adsorption of viral particles cannot be assured. It seems reasonable to assume that the percentage of infectious particles actually making contact with susceptible cells may be extremely low; the yield of lesser amounts of virus, therefore, is not unexpected.

With a viral inoculum of $10^6$ IU per ml or higher, the maximal titer attained approached that obtained with an intermediate dose, although, when the high inoculum was used, the peak was reached earlier on the third day of infection. At this time, over 50% of the cell population appeared nonviable. Subsequently, virus propagation diminished, and, within the next 3 days, the entire cell population was found to be nonviable. The establishment of a high nonviable cell population in a spinner culture infected with an inoculum of $10^6$ IU per ml or higher may be considered to be a type of cytopathogenic effect (cpe). This phenomenon may be related to the destructive cytopathology that occurred in monolayer cultures of many cell lines infected with a high inoculum of virus (Pirch and Mika, 1960). It is also reminiscent of the observation made.

**TABLE 3. Effect of medium changes on the growth of variola virus in spinner culture of guinea pig lung cells**

<table>
<thead>
<tr>
<th>Day after infection*</th>
<th>Virus titer</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Culture before centrifugation</td>
<td>Infected cells resuspended in fresh medium</td>
</tr>
<tr>
<td></td>
<td>IU/ml</td>
<td>IU/ml</td>
</tr>
<tr>
<td>0</td>
<td>$9.4 \times 10^4$</td>
<td>$3.8 \times 10^4$</td>
</tr>
<tr>
<td>1</td>
<td>$6.4 \times 10^4$</td>
<td>$1.1 \times 10^4$</td>
</tr>
<tr>
<td>2</td>
<td>$4.4 \times 10^4$</td>
<td>$1.2 \times 10^2$</td>
</tr>
<tr>
<td>3</td>
<td>$3.0 \times 10^2$</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Culture incubated at 35 C. Change of medium 199 + 20% horse serum accomplished on 2, 4, and 6 days.
by Mayyasi, Schuurmans, and Brown (1959) that $10^8$ or higher lethal doses of vaccinia virus were necessary to induce agglutination in monolayer cultures of L cells. These cells, although agglutinated, remained viable and continued to metabolize up to the third day (Brown, Mayyasi, and Officer, 1959).

It has been noted with inocula less than $10^5$ IU per ml that the peak titer of virus attained was generally proportional to the inoculated dose. This is not the pattern found in the usual bacterial or viral growth-curve studies, although delays may be evident when lower inocula are used, eventually equivalent peak titers are obtained. With inocula in excess of $10^5$ IU per ml, destruction of the host-cell population prevents proportionately greater yields of virus.

Intracellular replication of variola virus occurred early and rapidly in a spinner culture of guinea pig lung cells, although the liberation of virus into the suspending medium was a more gradual process. The use of larger inocula and a more efficient method of adsorption failed to affect peak titers attained. No effort was made to determine the amount of virus adsorption nor was any attempt made to demonstrate the presence of an eclipse phase. The latter phenomenon could only be expressed during the initial 6 to 7 hr postinfection and would require frequent and shorter time intervals for sampling and titration of disrupted cells.

It would appear that several complete medium changes tend to maintain a suitable environment for the infected cell culture resulting in fairly high and constant viral titers over a period of 7 days. The effect of medium changes on tissue cells infected with variola virus suggested that a close cell-virus association existed for at least 2 days in the culture. By the sixth day, a dissociation apparently occurred resulting in an increase of extracellular virus and a concomitant decrease in cell-associated virus. Whether this association under these experimental conditions truly involved intracellular virus instead of virus loosely bound or adsorbed to the cell membrane is presently not known.

**ACKNOWLEDGMENT**

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**LITERATURE CITED**


