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. PIERSCH. 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 $10^4$ 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), Vieuchange, deBrion, and Gueret (1958), Hahon and Kozikowski (1959), Marenikova, Gurwich, and Yemansheva (1959), and Piersch 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 Yamada strain of variola virus, characterized by Hahon, Ratner, and Kozikowski (1958), was used throughout these studies. A 10% suspension in heart infusion broth (Difco) was prepared from infected chorioallantoic membranes (CAM) as a fifth egg passage. This preparation had a titer of $1.4 \times 10^7$ infectious units (IU) per ml.

Tissue culture. The following cell lines were employed in these studies: guinea pig lung, Maben, embryonic human lung, L, and Chang human liver. Generally, the growth medium consisted of medium 199 containing 20% normal horse serum. The substrate for Chang human liver cells was 0.5% lactalbumin hydrolysate in balanced salt solution (Hanks) supplemented with 10% normal calf serum.

All spinner culture studies were carried out using an adaptation of the culture apparatus described by McLimans et al. (1957). The essential modification was the elimination of those accessories used for the CO$_2$ gas overlay. The spinner vessel was a standard, round-bottomed, 250-ml centrifuge bottle sealed with a rubber stopper perforated with (i) a sampling port covered with a vaccine stopper, (ii) a small air vent filled with cotton, and (iii) a small stainless steel rod supporting the magnet assembly. The use of a centrifuge bottle obviated the need of removing the culture from the apparatus during medium changes.

Procedures. In most experiments a spinner culture containing 100 ml of a cell suspension (approximately $10^6$ cells per ml) was inoculated with 1 ml of the virus preparation. An intermediate dose of virus ($10^3$ to $10^4$ IU per ml) was usually used as an inoculum although in certain experiments other virus-cell multiples were employed. An uninfected cell culture was always included to serve as a basis for comparing its physiological state with that of the infected culture during the course of each experiment. Appropriate daily samples were withdrawn from both infected and uninfected cell cultures for the determination of cell populations using the trypan-blue staining technique of Girardi, Michael, and Henle (1956). An additional aliquot from the infected culture was stored in a Dry Ice cabinet ($-35$ C) for subsequent titration on the CAM of 11- and 12-day-old embryonated chick eggs. A standard inoculum of 0.1 ml per egg was used and all data were expressed as infectious units per ml. The accuracy of the pock-counting technique coincided with the findings of Westwood, Phipps, and Boulter (1957).

For studies concerned with the growth kinetics of variola virus in a spinner culture of guinea pig lung
cells, 5 ml of egg seed virus (1.6 X 10^7 IU per ml) were added to 7.2 X 10^7 cells suspended in a minimal volume (15 ml) of complete medium and agitated for 1 hr at 35 C on a Magnamix. This was done to enhance adsorption with a virus multiplicity of 1. Infected cells were then centrifuged, washed twice with medium 199, and finally resuspended in 120 ml of the medium containing 20% normal horse serum. At this time (0 hr) and at designated intervals, samples were taken of the intact culture suspension, supernatant fluid, and cells that were centrifuged and washed an additional three times prior to sonic disruption. Cell disruption was carried out for 10 min in a Raytheon 9-kc sonic oscillator.

Results

Replication patterns of the virus. Five established cell lines were tested to ascertain their capacity to support the multiplication of variola virus in spinner cultures. A virus yield of 6.2 X 10^7 IU per ml was obtained with a guinea pig lung cell culture after its infection with 3.7 X 10^4 IU per ml (Table 1). The poorest growth response was evident with an L cell culture which produced only a tenfold increase of virus after 5 days.

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 10^4</td>
<td>6.2 X 10^5</td>
</tr>
<tr>
<td>Maben (CA of Human Lung)</td>
<td>Friesch et al. (1955)</td>
<td>1.0 X 10^4</td>
<td>4.1 X 10^5</td>
</tr>
<tr>
<td>Chang human liver</td>
<td>Chang (1954)</td>
<td>9.0 X 10^4</td>
<td>1.9 X 10^5</td>
</tr>
<tr>
<td>Embryonic human lung</td>
<td>Henle and Deinhardt (1957)</td>
<td>6.8 X 10^4</td>
<td>1.8 X 10^5</td>
</tr>
<tr>
<td>L (altered mouse fibroblast)</td>
<td>Sanford, Earle, and Likely (1948)</td>
<td>9.0 X 10^4</td>
<td>9.5 X 10^6</td>
</tr>
</tbody>
</table>

* Attained in 4 to 5 days.

Table 2. Growth of variola virus in spinner culture* of guinea pig lung cells infected with high inoculum

<table>
<thead>
<tr>
<th>Day after infection</th>
<th>Cell count (X10^6) infected culture</th>
<th>Per cent nonviable</th>
<th>Virus titer</th>
<th>Cell count (X10^6) uninfected culture</th>
<th>Per cent nonviable</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.6</td>
<td>&lt;1</td>
<td>1.1 X 10^4</td>
<td>0.5</td>
<td>2</td>
</tr>
<tr>
<td>1</td>
<td>0.6</td>
<td>&lt;1</td>
<td>1.8 X 10^4</td>
<td>0.7</td>
<td>&lt;1</td>
</tr>
<tr>
<td>2</td>
<td>0.6</td>
<td>9</td>
<td>3.5 X 10^4</td>
<td>0.9</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>0.7</td>
<td>50</td>
<td>1.0 X 10^7</td>
<td>1.9</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>0.6</td>
<td>80</td>
<td>8.3 X 10^4</td>
<td>1.8</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>0.7</td>
<td>87</td>
<td>9.0 X 10^4</td>
<td>1.8</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>0.6</td>
<td>100</td>
<td>9.0 X 10^4</td>
<td>1.9</td>
<td>1</td>
</tr>
</tbody>
</table>

* Medium 199 + 20% horse serum; incubation at 35 C.

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 10^4 to 10^5 IU per ml, were used to determine whether small amounts of virus would yield appreciable titers 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 10^6 IU per ml was employed, a maximal titer of 1 X 10^7 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 $5.0 \times 10^6$ 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 $10^8$ 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 $10^8$ 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

![Graph](image-url)
by Mayyasi, Schuurmans, and Brown (1959) that 10^5 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 demonstrate 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.

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LITERATURE CITED


