Retarded Growth of Poliovirus in Contact Inhibited Cells

By M. Fiszman, M. Reynier, Danielle Bucchini and M. Girard

Unité de Physiologie des Virus, Institut de Recherches Scientifiques sur le Cancer, B.P. n° 8, 94800 Villejuif, France

(Accepted 23 November 1973)

SUMMARY

CVI, BSC1, Vero, and primary monkey kidney cells were seeded either at low cell density and infected with poliovirus while growing, or at high cell density and infected under conditions of contact inhibition. Virus RNA synthesis and virus production were delayed in the cells seeded at high cell density, although neither adsorption nor uncoating of the parental virus particles were altered. In spite of the delay, however, virus RNA synthesis, once initiated, proceeded in the same fashion and at the same rate in both growing and resting cells. This implies that one of the first events following uncoating of the infecting virus was temporarily retarded in resting cells. There are indications that this delay might be due to a lag in the formation of parental virus polyribosomes.

INTRODUCTION

The growth of poliovirus is neither inhibited by 5 FUdR (Salzman, Lockart & Sebring, 1959) nor by amethopterin or 5 BUdR (Simon, 1961), indicating that cell DNA synthesis is not required. It is not inhibited by actinomycin D (Holland, 1962; Reich et al. 1961, 1962; Shatkin, 1962) except under particular conditions (Grado, Fischer & Contreras, 1965; Schaffer & Gordon, 1966; Cooper, 1966; and our unpublished results), indicating that cell RNA synthesis is probably not required either. Multiplication of picornaviruses occurs normally in cells in which cellular protein synthesis has been stopped (McCormick & Penman, 1968; Doyle & Holland, 1972). These results, as well as those obtained through the use of metaphase arrested cells (Johnson & Holland, 1965; Lake, Winkler & Ludwig, 1970; Bienz, Egger & Wolff, 1973), concur to show that the development of poliovirus is apparently independent from cell control.

We wish to report here on the effect of contact inhibition on the time course of poliovirus multiplication. As will be shown, virus development is delayed in contact inhibited cells, indicating that multiplication of the virus may, at least to some extent, be affected by controls of the host cell.

METHODS

The virus used was the Mahoney strain of poliovirus type 1. CVI and KB cells were grown without antibiotics in Eagle's minimal essential medium (MEM) supplemented with 10% tryptose phosphate, 1% glucose, and 5% calf serum (MCV1). Procedure for infection, labelling of the cells, analysis of virus RNA molecules, preparation of cytoplasmic extracts and analysis of polyribosomes have been described elsewhere (Fiszman et al. 1972).

Infectious centres assays. CVI cells were seeded in 5 cm plastic Petri dishes (Greiner,
Fig. 1. Influence of cell density on the time course of poliovirus multiplication. CV1 cells were seeded in 6 cm plastic Petri dishes at either $5 \times 10^5$ (○), $1 \times 10^6$ (×) or $2 \times 10^6$ (○) cells/dish and infected with $0.2 \text{ml}$ of poliovirus 24 h after seeding. Input multiplicity was in all cases adjusted to 50 p.f.u. cell. (a) Part of the cell cultures were labelled with $[^3$H]-uridine (0.5 μCi/ml, 25 Ci/mmol, C.E.A., Saclay) in the presence of actinomycin D (>5 μg/ml), and the incorporation of label into 5% TCA-precipitable material was determined as previously described (Fiszman et al. 1972). The results were expressed as ct/min/5 $\times 10^3$ cells. (b) At the indicated times, one Petri dish of each of the three cultures was frozen and thawed three times, and the virus titre was determined by plaque assay. The results were expressed as p.f.u./cell.

France) 24 h before infection. They were infected with $0.2 \text{ml}$ of a virus suspension, at an input multiplicity of 50 to 100 p.f.u./cell. After 30 min incubation at 36 °C, the cells were washed three times with phosphate-buffered saline (PBS) and detached from their support through the use of 0.005 % trypsin, $1.8 \times 10^{-4}$ M-EDTA. The number of infected cells was assayed by distributing dilutions of the suspension (1 to 100 cells) on to Petri dishes containing a preformed confluent monolayer of CV1 cells. After 1 h incubation at 36 °C the monolayers were covered with medium containing 0.75 % agar and the dishes were further incubated for 3 days, after which the number of plaques was scored.

RESULTS

The influence of cell density on the time course of poliovirus RNA replication

This was investigated in CV1 cells seeded at different cell densities 24 h previously. Virus RNA synthesis was followed by labelling the cells with $[^3$H]-uridine in the presence of
Effects of contact inhibition

Fig. 2. Influence of cell density on the time course of poliovirus RNA synthesis during the exponential phase of replication. CVI cells seeded at either $5 \times 10^5$ (●) or $2 \times 10^6$ (○) cells/dish were infected with poliovirus and treated with actinomycin D as described in the legend to Fig. 1. [H]-uridine ($5 \mu$Ci/ml) was added 30 min after infection. At the indicated times, cytoplasmic extracts in 0.01 M-tris-HCl, pH 8.5, 0.14 M-NaCl, 0.01 M-MgCl\textsubscript{2}, 0.5% Nonidet (NP 40) were prepared as previously described (Fiszman et al. 1972). All the cytoplasmic extracts were made 1% with respect to sodium SDS and centrifuged for 16 h at 27000 rev/min 20°C, in the SW 27 rotor of the Spinco, through 15 to 30% sucrose gradients in 0.01 M-tris HCl, pH 7.4, 0.1 M-NaCl, 0.002 M-EDTA, 0.05% SDS (SDS buffer). The amount of radioactivity sedimenting at 35 S was taken as a measure of virus RNA synthesized in each culture. This was plotted on a logarithmic scale as ct/min/10^6 cells versus time after infection.

actinomycin D. It was checked that, as with HeLa cells (Baltimore, Girard & Darnell, 1966), the majority of the incorporated radioactivity was in the 35 S poliovirus RNA. Fig. 1 (a) shows that the higher the cell density, the more delayed was the initiation of virus RNA synthesis. In cells seeded at $5 \times 10^5$ cells/Petri dish, the linear phase of RNA synthesis began at approx. the 3rd hour after infection, and lasted until approx. the 5th hour. This time is identical to that which has repeatedly been found with HeLa cells growing in suspension (Baltimore et al. 1966). In the cultures seeded at $1 \times 10^6$ or $2 \times 10^6$ cells/dish, the time course of RNA synthesis was delayed by approx. 1 and 2 h, respectively. Both the rate of virus RNA synthesis and the final amount of virus RNA synthesized per cell were, however, identical in the three cultures. This suggests that the lengthening of the virus growth cycle was not due to inhibition of virus RNA synthesis, but rather to delay in its initiation.

This was confirmed by analysing the time course of virus production in the three cultures...
Table 1. Percentage of infectious centres as a function of the number of cells per Petri dish

<table>
<thead>
<tr>
<th>Number of cells per Petri dish</th>
<th>$5 \times 10^5$</th>
<th>$1 \times 10^6$</th>
<th>$2 \times 10^6$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage of infectious centres</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>expt. 1</td>
<td>35</td>
<td>37</td>
<td>31</td>
</tr>
<tr>
<td>expt. 2</td>
<td>19</td>
<td>21</td>
<td>20</td>
</tr>
<tr>
<td>expt. 3</td>
<td>53</td>
<td>51</td>
<td>55</td>
</tr>
</tbody>
</table>

Fig. 3. Time course of poliovirus adsorption to either growing or resting cells. CV1 cells were seeded at either $5 \times 10^5$ (●) or $3 \times 10^6$ (○) cells/dish and infected 24 h later with 0.2 ml of purified [3H]-uridine labelled poliovirus ($1 \times 10^8$ ct/min/E$_{50}$). Input radioactivity was $2 \times 10^5$ ct/min/10$^6$ cells. At the indicated times, one Petri dish was withdrawn from each culture, washed twice with ice cold PBS, and cell-associated TCA-precipitable radioactivity was determined. The results have been expressed as percentage of input radioactivity recovered in each culture.

(Fig. 1 b). The final amount of infectious virus produced per cell was always the same, independent of the density of the culture, but the denser were the cultures at the time of infection, the longer was the lag in virus maturation.

The time course virus RNA synthesis during the early, logarithmic, phase of replication was then investigated. CV1 cells seeded at either $5 \times 10^5$ or $3 \times 10^6$ cells/Petri dish were infected at identical input multiplicities and the time course of 35 S virus RNA synthesis was determined in both cultures. Fig. 2 shows that the doubling time for virus RNA accumulation during the exponential phase of synthesis was the same in both types of cell cultures, but that replication of the virus genome was delayed by about 60 min in the confluent culture.

The influence of cell density on virus adsorption and penetration

This was investigated since differences in actual multiplicity of infection could have occurred owing to variations in the degree of virus adsorption and penetration, although
Effects of contact inhibition

Table 2. Adsorption and uncoating of poliovirus

<table>
<thead>
<tr>
<th>Time after infection</th>
<th>40 min</th>
<th>120 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cells per Petri dish</td>
<td>$5 \times 10^5$</td>
<td>$3 \times 10^5$</td>
</tr>
<tr>
<td>% input radioactivity adsorbed to the cells</td>
<td>9.2</td>
<td>9.2</td>
</tr>
<tr>
<td>% input radioactivity in encapsidated virus</td>
<td>1.2</td>
<td>1.07</td>
</tr>
<tr>
<td>% input radioactivity in 35S virus RNA</td>
<td>0.2</td>
<td>0.3</td>
</tr>
</tbody>
</table>

CV1 cells seeded at either $5 \times 10^5$ or $3 \times 10^6$ cells/Petri dish were infected with purified labelled poliovirus as described in the legend to Fig. 2. Input radioactivity was $2 \times 10^5$ ct/min/10^5 cells. At the indicated times, the cells were rinsed twice with PBS and scraped off the dish. Cell-associated radioactivity was determined on part of the cell suspension. A cytoplasmic extract was prepared from the other part. The extract was made 1% with respect to SDS and analyzed through 15 to 30% sucrose gradients in 0.01 M-tris HCl, pH 7.4, 0.1 M-NaCl, 0.001 M-EDTA, 0.5% SDS, for either 3 h at 25,000 rev/min, 20°C, for determination of radioactivity in encapsidated virus, or 16 h at 22,000 rev/min, 20°C, for determination of radioactivity in free 35S virus RNA (see reference Fiszman et al. 1972).

the cells were always infected with the same input multiplicity of infection. It is known that lowering the multiplicity of infection results in a lengthening of the virus growth cycle, but does not modify the doubling time for virus RNA synthesis during the logarithmic phase of replication (Baltimore et al. 1966).

The number of infectious centres in cells seeded at different cell densities and infected with an equal input multiplicity of infection was first determined (Table 1). No difference could be detected, whether the cells had been seeded at $5 \times 10^5$, $1 \times 10^6$ or $2 \times 10^6$ cells/dish. Only 20 to 50% of the infected cells registered as infectious centres. This is a surprisingly low yield, considering the high initial input (50 p.f.u./cell), but Hahn & Fogh (1970) have obtained comparable results.

Percentage of virus adsorption was next measured through the use of purified radioactive virus. Growing and confluent cells ($5 \times 10^5$ and $3 \times 10^6$ cells/dish, respectively) were infected with [3H]-uridine-labelled poliovirus at a multiplicity of 30 to 50 p.f.u./cell. Radioactivity associated with cell was determined after various times. The time course of virus adsorption was identical in both cultures, reaching a maximal value of 9 to 10% of the initial input within 45 min (Fig. 3).

In order to follow the fate of the labelled virus inside the cell, cytoplasmic extracts were prepared at 40 and 120 min after infection. They were analysed by sucrose gradient centrifuging in the presence of SDS (at neutral pH), and both the amount of encapsidated RNA and that of uncoated intact virus RNA molecules were determined. Cell-associated radioactivity was again 9% of the initial input, even at 2 h after infection (Table 2). Approx. 12% of this radioactivity was in encapsidated virus. Of the remainder, most was recovered as low mol. wt. material, and only 2 to 3% as intact 35S virus RNA molecules. These results confirm those reported by Joklik & Darnell (1961), who found that only approx. 0.4 and 1.5% of input poliovirus could be recovered as free intracellular virus RNA and intact virus particles, respectively. Table 2 also shows that no difference could be detected between resting and growing cell cultures, bearing either on the penetration or the decapsidation of the virus. Degradation of parental virus RNA was not increased in the confluent cell cultures. These similarities imply that the delay in virus RNA synthesis in the confluent cell cultures occurs after the uncoating of virus RNA.
Fig. 4. Formation of parental virus polyribosomes in growing and resting cell cultures. CV1 cells seeded 24 h previously at either $5 \times 10^5$ (●) or $3 \times 10^6$ (○) cells/Petri dish were infected with 0.2 ml of purified radioactive poliovirus ($6 \times 10^5$ cts/min/10⁴ cells). After 30 min adsorption at 36 °C the cells monolayers were rinsed twice, then incubated under 5 ml of MCV1 at 36 °C. Cytoplasmic extracts from both cultures were prepared 45 min later through Dounce homogenization in 0.01 M-tris HCl, pH 7.4, 0.01 M-NaCl, 0.0015 M-MgCl₂ (RSB). These were centrifuged in the SW 27 rotor of the Spinco for 3.5 h at 25000 rev/min, 4 °C, through 7 to 52 % sucrose gradients in RSB. Fractions were collected and assayed for TCA-precipitable radioactivity. The arrow labelled 74 S corresponds to the position of ribosomes as determined from the sedimentation profile of optical density. Cellular polyribosomes sedimented in fractions 19 to 25. The results have been expressed as cts/min/5 x 10⁵ cells.

Formation of parental virus polyribosomes in growing or resting cell cultures

Cells were again infected with radioactive poliovirus, and at 45 min after infection, cytoplasmic extracts were prepared and analysed by sucrose gradient centrifuging in order to determine the distribution of radioactivity between free virus RNA, virus particles and RNA-associated with polyribosomes. The sedimentation profile of the labelled material
Fig. 5. Virus development in KB cells seeded at different cell densities. KB cells were seeded at either $1 \times 10^6$ (○), $3 \times 10^6$ (•) or $6 \times 10^6$ (□) cells per plastic Petri dish and infected 24 h later with an input multiplicity of 50 p.f.u. of poliovirus/cell. Both the time course of virus RNA synthesis (a) and that of virus production (b) were then determined as described in the legend to Fig. 1.

extracted from the growing cell culture (Fig. 4a) showed three discrete peaks: one at the top of the gradient, probably representing degraded virus RNA molecules; the second at approx. 150 S, corresponding to still intact virus, and a third one sedimenting as virus polyribosomes (350 S). In the extract from the resting cell culture (Fig. 4b), the first two peaks were also present, but ‘little label’ was found in the position of virus polyribosomes. Cytoplasmic extracts prepared at 1.5 h after infection yielded similar results (not shown). Identical results were obtained in three different expts. Due to limitation in the amount of radioactivity, the labelled material recovered in the faster sedimenting fractions of the gradients shown in Fig. 4 could not be further characterized. These results nevertheless suggest that the formation of parental virus polyribosomes might be delayed in confluent cells, as compared to growing cells.

The influence of cell density on the time course of poliovirus RNA synthesis in KB cells

Similar results were also obtained with primary monkey kidney cells, BSC1 cells, and Vero cells (not shown). All these cell lines exhibit contact inhibition. The question arose whether tumour cells, which are known to be much less susceptible to contact inhibition (Abercrombie & Ambrose, 1962; Dulbecco, 1963; Berwald & Sachs, 1965) would behave in the same manner. Accordingly, KB cells were seeded at either $1 \times 10^6$, $3 \times 10^6$, or
Fig. 6. Uridine uptake in KB cells. KB cells were seeded at $1 \times 10^6$ (●) and $5 \times 10^6$ (○) cells/Petri dish. One day later, the cells were labelled with 0.05 μCi [14C]-uridine per ml. At the indicated times, the cells from one Petri dish in each culture were scraped off their support, washed 3 times with ice-cold PBS, and both TCA-soluble (a) and TCA-precipitable (b) radioactivities were determined. Results were expressed as ct/min/10⁶ cells.

$6 \times 10^6$ cells/Petri dish, corresponding to disperse, confluent, and highly confluent cultures respectively. At $6 \times 10^6$ cells/dish, the cells even formed small foci. One day after seeding, the cells were infected with equal multiplicities of poliovirus, and both the formation of virus particles and the synthesis of virus RNA were followed. Infectious particles were synthesized in an exponential manner, from approximately the 2nd to the 6th h after infection (Fig. 5 b). The time course of virus production and the final yield of virus particles per cell were in all instances identical, independent of the density of the cell culture.

The onset of virus RNA synthesis and the absolute amount of label incorporated was the same in all three cultures (Fig. 5 a). Therefore the amount of isotope incorporated is not proportional to the number of cells per Petri dish. This suggests that the penetration of uridine might be impaired in the more confluent cultures. The results in Fig. 6 show that such is indeed the case: both the maximal amount of uridine penetration into the acid-soluble pool of uninfected KB cells (Fig 6a) and its rate of incorporation into acid-precipitable material (Fig. 6b) differed by a factor of 3·5 between the least and the most confluent cultures. The results of Fig. 5 should therefore be corrected by an appropriate factor to take into account the decrease of uridine penetration into the more crowded KB cell cultures. Ultimately, this leads to the conclusion that cell density had no effect on the amount of virus RNA synthesized per infected KB cells. Unlike KB cells, CV1 cells did not show any effect of confluence on the rate of entry of uridine into the cell.

**DISCUSSION**

Both virus RNA synthesis and virus production were faster in growing than in contact-inhibited poliovirus infected cells. During the early, exponential phase of poliovirus RNA replication, the doubling time for virus RNA accumulation, was, however, identical in both
Effects of contact inhibition

Types of cell cultures. Similarly, RNA synthesis during the linear phase of virus RNA accumulation was independent of cell density, and the final amount of virus RNA synthesized per cell was identical, whether the cells were confluent or not at the time of infection. This shows that virus RNA synthesis was not slowed down in the contact-inhibited cell cultures, but that its onset was merely delayed. No differences could be found between the resting and the growing cell cultures in the extent and time course of virus adsorption, penetration, or uncoating. The observed delay must therefore result from the temporary inhibition of one of the very first events which occur upon uncoating, and before replication, of the infecting virus RNA molecules. The results obtained with confluent KB cells show that such inhibition is not related to high cell density per se, but to contact-inhibition of the cell culture in which infection takes place.

It is difficult to follow the fate of the parental virus RNA molecule(s) inside the infected cell. However, it can be assumed that one of the very first events which has to occur upon uncoating is the attachment of the parental RNA molecule(s) to host ribosomes. Of particular significance in this respect is the report that, in uninfected cells, mRNA attachment to ribosomes is much slower in contact-inhibited than in growing cells (Stanners & Becker, 1971). The observation reported above shows that, in the case of cells which were under conditions of exponential growth at the time of infection, some of the parental poliovirus RNA was found, shortly after infection, to sediment at the position of virus polyribosomes, whereas little if any was found in the case of contact inhibited cells. It could thus be that, in the case of the infected cell, the attachment of virus RNA to host cell ribosomes, resulting in the formation of the first virus polyribosome(s), would be slower in resting than in growing cell cultures. This partial and apparently temporary inhibition of translation of the virus message would then lead to delaying the onset of replication, through temporary lack of virus RNA polymerase. However, once this initial handicap is overcome, the kinetics of RNA replication, virus particle production and of virus protein synthesis are absolutely normal; whereas in uninfected confluent cells, the kinetics of protein synthesis are permanently retarded. Virus infection therefore partly releases the block of confluent cells.

Delays in the time course of virus development in contact-inhibited cells, as compared to that in growing cells, have been observed with numerous viruses other than poliovirus, and with DNA as well as with RNA viruses. For instance, both polyoma virus (Thorne, 1973) and SV40 (Manteuil et al. 1973) grow approximately twice as fast in growing as in resting cells. It would be tempting to think that the early step of virus multiplication which is affected by cell density in all these cases is common to both DNA and RNA viruses. The only known step they have in common is that of the formation of the first virus polyribosomes, either from the parental virus RNA molecules in the case of picornaviruses, or from the first molecules of early virus mRNA in the case of DNA viruses.

In any case, it is important to note that, whereas virus multiplication takes longer, and is less synchronous (Manteuil et al. 1973), in confluent than in resting cell cultures, the final yield of infectious virus per cell is identical in both types of cultures. It follows that, in practice, growing cells should usually be preferred for the study of virus events at the cellular and molecular levels, whereas resting cells can provide a better system for the growth and preparation of high titre virus stocks.

We thank Nicole Berthelot and Josette Merlino for their helpful technical assistance. This work was supported in part by the Commissariat à l’Energie Atomique and by the Direction des Recherches et Moyens d’Essais.
REFERENCES


(Received 11 July 1973)