The Mechanism of Interferon Action in Single Cells: accumulation of Intracellular Virus*

By TZE-TA CHANG†, E. H. SIMON and W. R. FLEISCHMANN, JUN.§

(Accepted 2 March 1973)

SUMMARY

Accumulation of intracellular mengovirus was studied in single, isolated mouse L cells. Pre-treatment with interferon delayed the initiation of virus replication in most cells, with the amount of delay varying widely from cell to cell. However, once the accumulation of intracellular virus began, it proceeded at an approximately normal rate. The data suggest that within a cell at any given moment, the action of interferon is usually all or nothing. That is, virus production is either totally inhibited, or free to proceed normally.

INTRODUCTION

In the accompanying paper (Fleischmann & Simon, 1973) single cells isolated in microdrops were used to investigate the effect of interferon on virus synthesis. In those experiments, mouse L cells were pre-treated with interferon, infected with mengovirus, and then distributed into microdrops which were sampled for the presence of virus at various times after infection. We concluded that the major effect of interferon was to delay the time of virus release. At increasing interferon doses, the delay became more marked, and an increasing proportion of the cells failed to yield virus at all.

Under normal conditions, mengovirus starts accumulating in L cells 5 to 8 h post-infection and is released as a single burst 1.5 to 2 h later. Virus accumulation in interferon protected cells is of some interest. There are two main possibilities; (a) virus synthesis starts at approximately the normal time, but proceeds slowly until lysis occurs, or (b) it starts late in infection, but proceeds at a normal rate. Distinguishing between these would shed considerable light on the mode of action of interferon both at the level of the individual cell and the whole organism. Under the first assumption, interferon would appear to affect many parts of the virus cycle, perhaps by causing a general reduction in virus protein synthesis (Kerr, 1971). The second assumption, however, implies a block at a single rate limiting step. At the level of the whole animal, if interferon merely served to slow the rate of virus production, then the small number of viruses appearing early in the cycle would probably be able to spread to surrounding cells through intercellular junctions (Johnson & Shendan, 1971) in advance of cell lysis. Under these conditions, interferon might well decrease the level of tissue virus titres without altering the number of infected cells. Indeed Koch (1964) has shown that the actual yield of virus has little influence on its spreading rate. But if interferon were to delay the time of virus synthesis, as well as the time of its release, its use as a protective agent would be greatly enhanced, since even a small delay would be magnified in

* Supported by grant no. GB 12623 from the National Science Foundation.
† Parts of this work were submitted in partial fulfillment of M.S. degree.
‡ Predoctoral fellow of the National Institutes of Health. Present address: Department of Microbiology, Indiana University.
successive virus growth cycles, and allow other body defence mechanisms to respond. Furthermore, the death of a fraction of the protected cells before they yielded would decrease the total number of viruses available for infection. Taken together, these factors would abort the infectious process.

To test the above possibilities, the accumulation of intracellular viruses in individual cells in the presence and absence of interferon was followed. Since the experiment involved the collection and titration of a large number of virus samples, improved methods of handling them were developed. The result of the present study supports the hypothesis that the accumulation of intracellular virus starts late, but once it begins, proceeds at a close to normal rate.

**METHODS**

**Cells and virus growth conditions.** L cell strain 929 was grown in 150 x 20 mm culture plates in Eagle's minimal essential medium (MEM Schwarz/Mann) with 10% newborn calf serum (Grand Island) and antibiotics. The same medium was used for virus growth. The mengovirus stock was the same heat resistant mutant used by Fleischmann & Simon (1973).

All experiments were carried out at 37 °C in a 5% CO2 atmosphere.

**Virus assay.** L cells grown in 50 x 15 mm plastic dishes (Falcon Plastics) were infected with mengovirus, absorbed for 45 min at 37 °C and covered with a starch overlay (Fleischmann & Simon, 1973). Plaques were counted 24 h post-infection after staining the monolayers with 1 ml 0.2% neutral red.

**Interferon preparation and treatment.** L cell interferon was prepared and assayed as previously described (Fleischmann & Simon, 1973).

**Superconditioned medium and microdrop technique.** The methods of Fleischmann & Simon (1973) were employed. Briefly, confluent plates of infected cells were trypsinized and diluted to about 500 per ml in superconditioned medium. The cells were then drawn into a spotting pipette, and microdrops set by touching the tip of the pipette to the bottom of a plastic Petri plate. The microdrops were covered with paraffin oil and each microdrop was mapped by examining it under the microscope and only those having single cells in them were used.

**Sampling and assay techniques.** The fluid of each microdrop was taken up in a capillary pipette, and dispensed into one cup of an RB-96 white 8 in × 12 in multidish plastic tray (Linbro) which contained 0.1 ml growth medium covered with a layer of paraffin oil to prevent evaporation of the medium. The capillary pipette was washed twice with the growth medium in the cup and then several times in a flask containing 100 ml growth medium. To save time, the same pipette was used to take all of the samples. A fresh capillary pipette was used to replace the medium into the microdrop. Intracellular virus was analysed by rupturing the cells by means of 2 freeze-thaw cycles. Control experiments showed that no virus was carried from one drop to the next by the above sampling technique.

**Multidish plaque assay.** 0.5 ml of an L cell suspension with a concentration of 1 x 10^6 cells per ml was distributed into each cup of a 8 in × 11½ in 96 CV-TC multidish plastic tray (Linbro) 8 to 24 h before infection.

To titre the microdrop samples, the fluid was removed from each cup and approximately equal amounts of each undiluted sample were distributed into six cups of a Linbro tray. The titre of each sample was obtained by summing the number of plaques.

These new procedures have resulted in substantial savings in terms of both time and cost.
Table 1. Effect of L cell monolayer cell concentration on plaquing efficiency of mengovirus

<table>
<thead>
<tr>
<th>Control</th>
<th>Number of cells/16 mm cup</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 x 10^4 cells/60 x 15 mm plate</td>
<td>2 x 10^5</td>
</tr>
<tr>
<td>Average plaque count</td>
<td>30.3 ± 9.5</td>
</tr>
<tr>
<td>Plaquing efficiency as % control</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 2. Effect of the volume of virus inoculum on plaquing efficiency

<table>
<thead>
<tr>
<th>Control plate 60 x 15 mm (200 λ)</th>
<th>Amount of virus fluid/cup (16 mm) (in λ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plaquing efficiency as % control</td>
<td>100</td>
</tr>
</tbody>
</table>

* An average of 60 cups in one experiment.
† An average of 95 cups in one experiment.
‡ An average of 4 experiments.
§ An average of 5 experiments.

The plaquing efficiency in each experiment is determined by the average number of plaques on many cups; the number of the cups varied from 20 to 50 in different experiments.

RESULTS

Multidish plaque assay

The standard procedure described in Methods were established by the following experiments:

(a) Optimum cell density. Cell concentrations were varied from 2 x 10^5 to 5 x 10^5 per cup. A sample of 0.1 ml mengovirus was distributed evenly on to six cups of a Linbro tray overlayed as described previously, and counted 24 h later. The titre, determined by summing the plaques on the six cups, was compared to that from a control experiment which was performed on standard plates.

Each plaque count in Table 1 represents an average of eight sets of six cups. The plaquing efficiency was evidently not sensitive to variations in monolayer cell concentrations within the range examined. The concentration of 5 x 10^5 cells/cup which corresponds to 4 x 10^6 cells/standard plate, was used throughout this project.

(b) Optimum vol. of virus inoculum. In the experiments summarized in Table 2, a constant number of p.f.u. were added to the cups in the indicated amount of fluid. The results show that the plaquing efficiency increased with decreasing vol. of inoculum. In the plaque assay as described in Methods, a 0.1 ml sample was evenly distributed on to six cups. This procedure represented a reasonable compromise between ease of handling the sample and maximum plating efficiency. The final assay is about 70% as efficient as the standard Petri dish assay.

Interferon effects of virus production in mass culture

The effect of interferon on virus production depends both on the interferon concentration and on the multiplicity of infection (Hallum & Youngner, 1966; Takemoto & Baron, 1966; Fleischmann & Simon, 1973). Other factors such as growth temperature and cell age may also affect the interaction between interferon and cells (Morahan & Grossberg, 1970). But whatever the quantitative effect on virus production, interferon always causes a delay in the time of maximum yield and a decrease in the amount of virus produced.
Fig. 1. Intracellular and extracellular mengovirus production in normal L cells. Several plates of L cells were infected with mengovirus at an input multiplicity of 17 p.f.u./cell as described in Methods. At various sampling times, the fluid from three plates was collected and pooled to measure extracellular virus. Two ml of growth medium was then added back, and the cells were lysed by one freeze-thaw cycle. These fluids were then pooled as an intracellular virus sample. The average yield was 250 p.f.u./cell. O--O, extracellular viruses; •--•, intracellular viruses.

Fig. 2. Intracellular and extracellular mengovirus production from interferon-treated L cells. Procedures were as described in the legend to Fig. 1, except that all the plates were pre-treated with 8 units of interferon for 12 h before challenging with mengovirus. The average yield was about 21 p.f.u./cell. O--O, extracellular viruses; •--•, intracellular viruses.

The data of Fig. 1 compare intracellular and extracellular mengovirus production in cells not treated with interferon. The intracellular virus concentration reached a maximum at 8 h post-infection, while extracellular virus reached its peak at 10 h. Note that at 6 h, the intracellular virus was at least tenfold greater than extracellular, suggesting that virus was accumulated in the cell and then released as a burst, beginning at about 8 h after infection. Subsequent work using microdrops confirmed this conclusion. Fig. 2 similarly displays the intracellular and extracellular virus accumulation in cells pre-treated with eight units of interferon. Comparing Figs. 1 and 2, three major differences may be noted. First, the final amount of virus from the interferon-treated cultures was about tenfold lower than from the control. Secondly, the rise of the extracellular virus concentration was slower in the interferon-treated culture, and maximum yield not reached until 14 h after infection, compared to 8 to 10 h in the controls. Thirdly, the rate of decrease of intracellular virus was much slower in the interferon-treated cultures. Similar results have been obtained in numerous
Accumulation of intracellular virus

Fig. 3. Kinetics of extracellular mengovirus production by mass cultures of L cells treated with various amounts of interferon. Sixty plates of L cells were divided into three groups, one of which was overlayed with 4 units interferon (○—○), one with 8 units (△—△), and the third with growth medium (●—●). After 12 h, the fluid was removed, and the plates washed and infected with mengovirus at an input multiplicity of 10 p.f.u./cell. At various sampling times, the fluid from two plates was pooled and titred. Final yield was control = 300 p.f.u./cell; 4 units = 50 p.f.u./cell; 8 units = 15 p.f.u./cell.

Fig. 4. Effect of trypsinization on the kinetics of virus production in the presence and absence of interferon. Four control plates and four interferon plates which were pretreated with 8 units interferon for 12 h were infected with mengovirus at an input multiplicity of 40 p.f.u./cell. Three h later, two plates from each set were trypsinized. The cells were resuspended into 2 ml growth medium and put on to standard plates. At the same time, the other four plates were washed with buffer and 2 ml growth medium was added back to each of them. At various times, samples were taken by withdrawing 0.1 ml growth medium. ●—●, control; ○—○, control-trypsinized; △—△, interferon; △—△, interferon-trypsinized.

other experiments. It is not clear from these data if the intracellular virus formed in treated cells resulted from a slow accumulation in all cells, or if at any given moment, a fraction of the cells was producing intracellular virus at nearly optimal rates. A third possibility, that protected cells continually produce and release virus, has been eliminated by the work of Fleischmann & Simon (1973).

The effect of interferon on the time of virus yield is demonstrated by the data of Fig. 3. The results of experiments similar to those of Figs. 1 and 2 are presented as the percentage of final yield as a function of time after infection. In the control the maximum yield was attained at 10 h post infection, but was delayed to 16 and 20 h for cells treated with four and eight units of interferon respectively.
Table 3. Sampling time of microdrop experiment

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass culture</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Extracellular only</td>
<td>C†</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Control§</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Interferon§</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
</tbody>
</table>

* Hours post-infection. †Control (group B). ‡Interferon treatment (group A). § Both intracellular and extracellular viruses were sampled. × Samples were taken.

Fig. 5. Procedures of a microdrop experiment. Detailed procedures are in the text. Each set of microdrops is sampled for both intracellular and extracellular viruses at various times as given in Table 3.

Preliminary controls for microdrop experiments

As described in Methods, cells were infected, trypsinized, and then placed in a microdrop. The data of Fig. 4 show that trypsinization of cells after infection does not alter their virus production. Another technical problem was that the cells had to be kept at room temperature for about 3 h during the setting and mapping procedure. Since this much time at room temperature caused a delay in virus production (Chang, 1972), a mass culture was included along with each microdrop experiment, and subjected to the same temperature shifts as the single cells.

Growth of mengovirus in isolated L cells

Six confluent L cell plates were divided into two groups. Group A was overlaid with six units of interferon and group B with growth medium. Twelve h later, three plates from each group were infected with mengovirus at an input multiplicity of 40 p.f.u./cell. Microdrops were set and mapped as described in Methods. Twelve microdrops selected from both interferon-treated and control groups were sampled periodically for extracellular viruses by removing
Fig. 6. The fraction of control and treated cells containing intracellular virus at various times after infection. Control and interferon-treated L cells were infected and individual cells placed into microdrops as described in Methods. At various times, 12 microdrops with interferon-treated cells and 8 with control cells were sampled for their intracellular virus content. Microdrops with untreated cells were sampled for 25.5 h. Detailed procedures are given in the text. The percentage of cells containing intracellular virus at each sampling time is shown. □, control; □, interferon-treated cells.

Fig. 7. The percentage of control and interferon-treated single cells containing extracellular virus at various times after infection. Procedures as in Fig 5.
the medium and then adding back new superconditioned medium. One hundred and forty-four microdrops selected from group A were divided into 12 sets of 12 microdrops each, and 64 microdrops from group B were divided into eight sets of eight microdrops each. Those microdrops were sampled by collecting their intracellular and extracellular viruses at various times. Procedures of harvesting virus are summarized in Table 3 and Fig. 5.

It can be seen in Fig. 6 that at 10 h post-infection, 100% of the control microdrop samples contained intracellular virus. As expected, after 10 h, the number of control cells with intracellular virus dropped rapidly. Only 58% of the interferon-treated cells, however, contained intracellular virus at 12 h, and the number dropped slowly from 12 to 26 h, indicating either retention of virus by some of the cells, or appearance of cells containing newly synthesized intracellular virus. The data of Fig. 7 show that 7 out of 8 control microdrops contained extracellular virus at 12 h, while the number of interferon treated ones containing extracellular virus rose steadily from 12 to 26 h post-infection. Nonetheless, at 26 h, 5 out of 12 treated microdrops still contained neither intra- nor extracellular virus. Hence, while interferon generally delays the time of virus production, the degree of delay varies greatly from cell to cell. Fig. 8 illustrates that at early times, those interferon-treated cells which contained intracellular viruses often contained a substantial amount of them. Experiments by Fleischmann (1972) employing many more cells show that at 11, 14 and 17 h post-infection the yield from cells treated with 4 units of interferon was at least two-thirds that of the controls. Therefore, virus made in those cells must have accumulated at least half the normal rate. Furthermore, in those cells that yielded later, the number of intracellular viruses did not increase over time. On the contrary, as shown in Fig. 8, the average virus content decreased slightly as a function of time, suggesting also that the period of virus accumulation is not prolonged by the interferon treatment. The yield of extracellular virus in each microdrop as illustrated in Fig. 9 supports these conclusions.
DISCUSSION

Studies of virus development employing mass cultures of infected cells necessarily average millions of individual events, and as a consequence those phenomenon involving only a fraction of the population might not be detected. This difficulty can be avoided by using single cells isolated in microdrops. Fleischmann & Simon (1973) showed that an individual cell 'bursts' over a 15 to 20 min period whether protected by interferon or not. However, whereas most normal cells lysed 11 h after infection, in protected cells lysis occurred from 11 to 26 h after infection, depending on the interferon concentration and the input multiplicity. Initiation of virus replication was delayed in most of the cells (Figs. 6 and 7), but once synthesis began, virus accumulated fairly rapidly (Figs. 8 and 9).

Fleischmann & Simon (1973) proposed that cells lysing later than 12 h post-infection had deteriorated to the point where they were either no longer capable of supporting virus production at a full rate, or more likely, for the normal period of synthesis. Premature rupture of cellular lysozomes may be involved in this process (Lwoff, 1969; Guskey, Smith & Wolff, 1970).

The relatively rapid accumulation of intracellular virus after the inhibition is released suggests that virus production is usually either totally inhibited or free to proceed more or less normally. Otherwise there would be a prolonged period during which virus would slowly accumulate, and this was not observed (Fig. 8). These considerations suggest that the antiviral protein (AVP) whether it is induced by interferon (Taylor, 1964) or is interferon itself (Sheaff & Stewart, 1969) blocks a single critical step in virus synthesis, and that once this is overcome, synthesis can proceed at a normal rate. In agreement with work on mass cultures, the average delay in burst time depended upon both the concentration of interferon with which the cell is treated, and on the input multiplicity, but is independent of whether or not interferon is present during infection, i.e. the antiviral protein does not decay appreciably during the course of the experiment (Fleischmann & Simon, 1973).
The mode of action of interferon is presently unclear, with some evidence arguing that it primarily inhibits translation (Kerr, 1971) and other data suggesting that its major site of action is transcription (Marcus et al., 1971). Our own results are consistent with several models. In one type, the AVP is in competition with a cellular component—possibly one involved in translational control. While a second class assumes that the virus itself can produce (or induce) an anti-interferon agent (for example, the stimulon described by Chany, Fournier & Roussett, 1970), which might accumulate even in the presence of AVP, and eventually increase its concentration to a level where RNA synthesis can commence. The following facts are consistent with both models: (a) A high input multiplicity can overcome interferon action. (b) The variation in time of initiation of virus synthesis is much greater in interferon-protected cells than in normal cells; and (c) certain viruses, such as SV5, can cause a persistent infection in otherwise healthy cells (Choppin, 1964). These viruses are sensitive to interferon when the cells are treated before infection, but resistant once the infection has been established. Furthermore, cells infected with these viruses do not produce interferon, and cannot assume an antiviral state with respect to superinfecting viruses (Choppin, 1964). A detailed description of these models would be premature, but they do indicate how several otherwise difficult to comprehend phenomena may be explained.

Finally, by showing that in treated cells virus does not accumulate until late in infection, our results help explain the action of interferon in the whole animal. Virus accumulation at normal rates, even in the absence of lysis, might still permit the rapid spread of virus by means of intracellular connexions (Johnson & Shendan, 1971). But since both extracellular and intracellular accumulation are inhibited, the seemingly small delay in virus production will be magnified in successive cycles, and allow the body’s other defenses such as fever and the various immunological responses to overcome the infection (Lwoff, 1969). Furthermore, the total viraemia is reduced because a substantial fraction of the treated cells never release virus. Taken together, these factors would lead to the termination of the virus infection.

The authors wish to acknowledge the excellent technical assistance of Miss Jennifer Leuck.

REFERENCES

Accumulation of intracellular virus


(Received 11 October 1972)