Effect of Interferon on Virus Production from Isolated Single Cells*

By W. R. FLEISCHMANN, JUN.† AND E. H. SIMON‡

Department of Biological Sciences
Purdue University, West Lafayette, Indiana 47907, U.S.A.

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SUMMARY

Microdrops were used to isolate interferon treated mouse L cells infected with mengovirus. Three parameters were measured, (1) the time of virus yield, (2) the yield/cell, and (3) the percentage of yielding cells. More than 97% of the control single cells yielded an average of 167 p.f.u./cell with most of them yielding between 11 and 14 h post-infection. Cells treated with interferon yielded later than controls (if at all), and all could be protected to some degree. In both treated and control cells, virus was released over a period of about 15 min. Increasing the multiplicity of infection of challenge virus reduced the effectiveness of a given interferon dose, while increasing the concentration of interferon increased the length of the latent period, decreased the size of the yield, and decreased the number of yielding cells. On the basis of these observations it is proposed that (1) the direct effect of interferon protection is to delay a critical step in virus synthesis, (2) the reduction in yield size and in the number of yielders is due to a progressive loss of the inherent ability of the cell to produce virus, and (3) the delay is the result of interferon, or a molecule induced by it, acting as a competitive inhibitor of some cellular or virus product, needed for the completion of virus synthesis.

INTRODUCTION

It is not known if interferon inhibits virus production by all cells equally. Reductions in virus yield range from slight to considerable (Ho, 1962; Levine, 1962; Lockart & Horn, 1963; Levine et al. 1967; and Gandhi & Stewart, 1969). In 1961, Wagner found that interferon treatment lengthened the latent period of eastern equine encephalomyelitis virus. This observation has been confirmed (Mayer, Sokol & Vilcek, 1962) and extended to include a variety of viruses: Semliki Forest virus (Taylor, 1965); encephalomyocarditis virus (Takemoto & Baron, 1966); adenovirus type 2 (Gallagher & Koobyraian, 1969); and vaccinia virus (Gifford & Ghosh, 1965; Joklik & Merigan, 1966). Thus, interferon both delays the time of virus production and reduces the yield.

Since monolayer techniques can only measure average yields, and since infectious centre assays are subject to considerable error, a microdrop technique permitting the rapid isolation of single cells in individual drops of medium was used to investigate the effect of interferon on virus production by individual cells.

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† Predoctoral fellow of the National Institute of Health, present address, Department of Microbiology, Indiana University.
‡ Direct reprint requests to Edward H. Simon.
Methods

Cells and virus. L cells, strain 929, (mouse fibroblasts), obtained from the Dow Chemical and Pharmaceutical Company (Zionsville, Indiana), were maintained at 37 °C in a humidified, 5% CO₂ atmosphere on 150 mm glass Petri plates and routinely passaged every 4 to 7 days. For plaque assays and microdrop experiments, cells were seeded on 60 mm plastic Petri plates (Falcon Plastics). Unfortunately, the original line of L cells became contaminated and was replaced. Although the new line came from the same source, it differed slightly from the old one in that the latent period for virus production was one h longer than in the old cell line. Since the results were otherwise comparable, for purposes of clarity, all data are expressed relative to the harvest times for the newer cell line.

Mengovirus, supplied by Dr Richard Franklin, was selected for resistance to heat decay at 37 °C and plaque purified. Stocks were prepared by infecting L cell monolayers on 150 mm glass Petri plates. About 20 h post-infection, the cells were disrupted by three cycles of freeze-thaw and the media was collected, spun at low speed to remove coarse cell debris, distributed in 1 ml samples, and stored at -60 °C. Titres averaged 10⁶ p.f.u./ml.

Assay system. Mengovirus was assayed by a plaque assay system similar to Dulbecco's (1952). Day-old monolayers of approximately 4 x 10⁶ L cells were overlaid with 0.2 ml of virus diluted in growth medium, incubated at 35 °C for 45 to 60 min to allow virus adsorption, and covered with 5 ml of a starch overlay medium adapted from DeMaeyer & Schonne (1964). The monolayers were incubated at 37 °C for 24 to 28 h in a humidified, 5% CO₂ atmosphere before staining with 1 ml of an 0.2 700 neutral red (w/v) solution in phosphate diluent. Plaques were counted two h later.

Preparation of starch overlay. A quantity of starch (18.5 g) (Starch hydrolysed, Schwarz/Mann Research Laboratories) was dissolved in 40 ml sterile water and then added slowly with vigorous stirring, to a 500 ml flask containing 110 ml boiling water. Just as it reached the boiling point, the starch solution was removed from the hot plate and allowed to cool to 60 °C at room temperature, at which time, a mixture of 37 ml 5 x Eagle's (medium with 5 times the normal concentrations of salts and amino acids) and 15 ml newborn calf serum at 48 °C was added. The temperature of the overlay when added to the cells was about 50 °C. All details of this procedure are quite critical, but the optimum starch concentration varies for different batches.

Media. L cells were grown in Eagle's minimal essential medium (Schwartz-Mann) supplemented with 10% newborn calf serum (Grand Island Biological). Single cells were incubated in a 'super-conditioned' medium prepared by exposing 3- to 5-day-old confluent stationary phase L cell monolayers on 150 mm Petri plates in 15 ml of growth medium to u.v. light from a 15 W germicidal lamp (GE) at a distance of 50 cm for 20 min. The cells were incubated for 48 h, and then the supernatant fluid was harvested, clarified by centrifuging at 14,500 rev/min for 15 min and stored at 4 °C. Conditioned medium has been shown to enhance the growth of small numbers of cells (Fisher & Puck, 1956; Eagle & Piez, 1962; Rubin, 1966) with the greatest enhancement obtained when the superconditioned medium was obtained from stationary phase cells (Takahashi & Okada, 1970). Adequate virus yields from single cells were obtained only when this medium was used.

Interferon preparation and titration. Interferon was prepared by infecting L cell monolayers of approximately 4 x 10⁶ cells on a 60 mm Petri plate with Newcastle disease virus (NDV) at an input multiplicity of 30 p.f.u./cell. Twenty-four h later, the supernatant fluid was collected, centrifuged at low speed, dialysed at pH 2 for 12 h at 4 °C in a 0.05 M-KCl solution (pH adjusted with HCl) redialysed against phosphate buffer pH 7.2 and stored at

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4 °C. Treatment at pH 2 reduced the original NDV titre in the interferon preparation from 10^5 p.f.u./ml to undetectable levels. The crude preparation was not purified and was generally used at dilutions of 50 or more to minimize the influence of impurities. To confirm that residual NDV was not inducing additional amounts of interferon, the preparation was added to monolayers at the levels employed in actual experiments. When the supernatant fluids were subsequently titrated, no increase in interferon titre was observed. Furthermore, the physical particle multiplicity in such experiments was always less than 0.1/cell.

Interferon titres were determined by a plaque reduction assay similar to Wagner's (1961). L cell monolayers of 2 × 10^6 cells on 60 mm Petri dishes were overlaid with 2 ml of various dilutions of interferon in growth medium and incubated for 10 to 12 h. The monolayers were challenged with 100 to 200 p.f.u. of mengovirus/plate and plaques were counted at precisely 30 h post-infection, when they were about 1 mm in diameter. Titres were expressed as the reciprocal of that dilution, in 2 ml, which reduced plaque numbers to 50% of the control and generally ranged from 800 to 1600 units. Ten units of 2 separate batches of NIH standard mouse interferon titred at 1 ± 0.2 units by this assay.

Isolation of single cells. A spotting pipette was calibrated by dispensing a given vol. drop-wise on to the surface of a plastic Petri dish. Monolayers of L cells were trypsinized and diluted in super-conditioned medium to give an average of one cell from each drop (about 2 μl). Sixteen microdrops were then dispersed on the surface of a 60 mm plastic Petri plate and overlaid with paraffin oil which served both to stabilize the drops and to prevent them from drying. They were then examined microscopically to determine which ones contained a single living cell. As anticipated, about 35% of the microdrops met this criterion. After about 2 h of incubation at 37 °C, cells were firmly attached to the Petri plate and fluid could be added to, or removed from, the microdrop with a drawn glass capillary pipette without disturbing the cell.

Virus production by single cells. Monolayers containing approximately 3 × 10^6 cells were infected with 0.2 ml mengovirus in growth medium, incubated for 45 to 60 min to allow virus adsorption, overlaid with growth medium, and reincubated. Three h post-infection, both plates were washed 3 times with phosphate diluent, and while one plate was used for microdrops, the other was refed with growth medium. (Trypsinization at 3 h post-infection had no effect on the virus growth cycle, but trypsinization before 2 h or after 4 h post-infection reduced both the size of the yield and the number of yielding cells.) The infected cells were dispensed into microdrops as described above. The control monolayer and the microdrops containing single cells were then incubated, harvested at the appropriate times, reoverlaid with medium, and incubated again. This cycle was repeated at three h intervals from 11 h to 26 h post-infection. Control experiments showed that virtually no virus yields occurred after 26 h post-infection. An identical procedure was followed with interferon-protected cells, except that those monolayers were incubated with varying concentrations of interferon for 10 to 12 h before infection.

Microdrops were harvested as follows: (1) the microdrop was entered at the upper right-hand margin with a drawn glass capillary attached to a bulb; (2) the bulk of the fluid was removed from the microdrops leaving only a film of medium on the surface of the Petri plate indicating where the microdrops had been, and deposited in a test tube containing 0.5 ml growth medium; (3) the capillary was rinsed twice in the test tube medium and twice in an Ehrlenmeyer flask containing 100 ml of Eagle's medium. Since this procedure reduced virus carryover to less than 0.1%, the same pipette was used to harvest all the drops; (4) using a fresh capillary pipette, medium was returned to the microdrops by allowing a drop of medium to form at the tip of the capillary and then lowering it through the paraffin oil until
it touched the upper-right-hand margin of the microdrop, and the drop coalesced with the film of medium remaining from the old microdrop. With practice, over 100 microdrops/experiment could be handled this way.

RESULTS

Growth curve of mengovirus in control and protected L cell monolayers

The data of Fig. 1 show that treatment with 4 units of interferon reduced virus production by one-half (31 p.f.u./cell compared to 67 p.f.u./cell) and greatly delayed the time at which virus was yielded. In control monolayers, most virus was synthesized between 7 and 10 h post-infection and virus release was virtually complete by 12 h post-infection. A comparison of the extra and intracellular virus concentrations has lead to the conclusion that mengovirus is released as a burst (Franklin, 1962). In interferon treated monolayers, virus production was delayed by about 5 h, as significant levels of virus production were not reached until 11 h post-infection and virus synthesis continued beyond 17 h post-infection.

Efficiency of single cell isolation

When a suspension of cells is divided into microdrops of equal vol., they are expected to be distributed according to the Poisson distribution: \( P(r) = \frac{m^r e^{-m}}{r!} \) where \( m \) is the average number of cells/microdrop, \( e \) is the Napierian logarithm base, and \( r \) is the actual number of cells in a microdrop. In almost every experiment this expectation was confirmed. However, while 35% of the microdrops contained a single cell, 6% of those (or 2% of the total) were not chosen because the single cell was located at the upper right-hand margin of the microdrop, and thus about 1/3 of the total microdrops contained a single cell suitable for our experiments.

Efficiency of harvesting microdrops

The possibility of virus inactivation by paraffin oil or by adsorption to the surface of the Petri plate, was investigated by placing equal vol. (about 0.01 ml) of a virus dilution either onto a Petri plate, or into a test tube containing growth medium. The drops were overlaid with paraffin oil and harvested at various times. No virus was lost. Another experiment demonstrated that more than 99% of the virus present in a microdrop was recovered in a single harvest.

Titration of interferon

While 4 units of interferon reduced one-step virus yields from mass cultures by only 50% (see Fig. 1), the same amount reduced the number of plaques in a plaque inhibition assay to 4% of the control level. This difference is understandable because plaque formation is the result of multiple growth cycles. Therefore, even though most cells are producing virus, the cumulative delays induced by interferon slow plaque development to the point where they are not visible as soon as those on control plates.

Virus production by single cells

Effect of the microdrop technique on virus production

Repeated washing of the microdrops had no effect on the time or size of later virus yields provided that the microdrops were first incubated long enough to permit cell attachment. To further test the method, 217 single cells distributed over several experiments were sequentially harvested. The average virus yield of the 217 which yielded virus compared favourably with the average virus yield/cell in monolayer culture (range of 55 to 285 with overall average of 190 for monolayers). Furthermore, Fig. 2 compares the one step growth
Fig. 1. Comparison of one-step growth curves of mengovirus in interferon treated and control monolayers. L cell monolayers containing approximately $3 \times 10^6$ cells/plate were incubated with 2 ml of medium containing either (A) 0 or (B) 4 units of interferon for 11 h and then challenged with 16 p.f.u./cell. Samples were collected from separate plates at the indicated times and assayed for released virus. *Intracellular* virus was determined by adding back fluid to each of the plates (after three washes to remove residual released virus), and freeze-thawing three times. The yield/cell was 67 for control monolayers and 31 for treated monolayers. ●—●, total virus; ○—○, released virus.

Fig. 2. One-step growth curves of mengovirus in interferon treated and control single cells and monolayers.

(A) *Microdrops*. L cell monolayers were incubated with either 0 or 4 units of interferon for 11 h. The monolayers were challenged with 30 p.f.u. of mengovirus/cell. At three h post-infection, the cells were trypsinized and dispensed at an average of one cell/microdrop. In the course of three experiments, a total of 45 control and 104 treated microdrops were sequentially harvested at the indicated times. No harvests were made after 26 h because control experiments demonstrated that virtually no bursts occurred after that time. Single cell yields averaged 131 p.f.u. for controls and 99 p.f.u. for treated cells. The range and average value for the three experiments are indicated on the graph. ○—○, control; ●—●, + interferon.

(B) *Monolayers*. Monolayers were treated with interferon and infected as above. The supernatant fluids were harvested and replaced at the indicated times. Control yields averaged 125 p.f.u./cell and interferon treated cells averaged 62 p.f.u./cell.

curves of mengovirus in single cells and in monolayers, and demonstrates that the microdrop technique did not appreciably alter the time course of virus release. This has been confirmed in a large series of microdrop experiments. Thus, the manipulations involved in the isolation of single cells and their subsequent harvesting did not significantly affect the mengovirus infectious cycle in either control or protected cells.
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**Fig. 3.** Virus yields by single interferon treated and control cells at various times post-infection.

L cell monolayers were incubated with 4 units of interferon as in Fig. 1, challenged with 45 p.f.u. of mengovirus/cell and dispensed into microdrops as described for Fig. 2A. 15 control and 35 interferon microdrops were serially harvested at 11, 14, 17, 20, 23 and 26 h post-infection. The cumulative distribution of virus yields is presented for 11 (A), 14 (B), 17 (C) and 26 (D) h post-infection. The average control yield was 135 p.f.u./cell and the total average yield from treated cells was 95 p.f.u./cell. Since all of the control cells had yielded at the 11 h harvest, their cumulative distribution did not change, and for purposes of clarity, the distribution is shown only at the first harvest time (A). □, control; □ + interferon.

**Analysis of virus production by interferon protected cells**

The pattern of virus production from protected cells was determined by harvesting the microdrops periodically from 11 to 26 h post-infection and plotting the cumulative yields. Fig. 3 shows the results of one experiment which is representative of sixteen others employing varying amounts of interferon and different multiplicities of infection. All of the control cells yielded by 11 h post-infection; the distribution is shown in graph A. Three aspects of the yields from treated cells should be noted: (1) virtually every L cell in the population responded to a low level of interferon, (2) the proportion which yielded increased with time, and (3) the distribution of yields changed with different times post-infection. The profile in graph C might suggest an all-or-none effect of interferon, since there is a high proportion of non-yielders and a nearly normal distribution of yields around the control average, while graph D might suggest a graded effect. The increase in low yielders in graph D relative to graph C is reproducible, and is discussed below.

Table 1 summarizes seventeen separate experiments representing a total of 217 control and 533 protected single cells. More than 97% (211/217) of the control single cells yielded virus, demonstrating the efficiency of the technique. 95% (28/294) of those protected cells which produced virus, and 81% (17/211) of the controls yielded virus at two harvest times. These split bursts probably represent microdrops which were harvested during the period of virus

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Table 1. Summary of virus yields from interferon protected and control single cells

<table>
<thead>
<tr>
<th>Hours post-infection</th>
<th>Control</th>
<th>½</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>16</th>
<th>32</th>
<th>64</th>
<th>128</th>
</tr>
</thead>
<tbody>
<tr>
<td>11†</td>
<td>98 (142)</td>
<td>80 (4)</td>
<td>74 (6)</td>
<td>75 (27)</td>
<td>32 (13)</td>
<td>37 (2)</td>
<td>68 (1)</td>
<td>63 (53)</td>
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<tr>
<td>12'5§</td>
<td>130 (15)</td>
<td>99 (11)</td>
<td>62 (5)</td>
<td>131 (1)</td>
<td>90 (17)</td>
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<td>14</td>
<td>103 (45)</td>
<td>54 (21)</td>
<td>99 (8)</td>
<td>66 (5)</td>
<td>47 (3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15'5</td>
<td>97 (2)</td>
<td>67 (1)</td>
<td>84 (3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>44 (5)</td>
<td>37 (10)</td>
<td>76 (5)</td>
<td>181 (1)</td>
<td>18 (7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18'5</td>
<td>66 (1)</td>
<td>50 (3)</td>
<td>32 (41)</td>
<td>54 (2)</td>
<td>54 (2I)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>138 (1)</td>
<td>63 (5)</td>
<td>23 (2)</td>
<td>48 (12)</td>
<td>32 (1)</td>
<td>32 (1)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>21'5</td>
<td>25 (5)</td>
<td>66 (3)</td>
<td>24 (7)</td>
<td>69 (2)</td>
<td>73 (2)</td>
<td>18 (3)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>64 (1)</td>
<td>64 (1)</td>
<td>64 (1)</td>
<td>64 (1)</td>
<td>64 (1)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>24'5</td>
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<td>13 (4)</td>
<td>15 (1)</td>
<td>39 (2)</td>
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<td>21 (9)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>32 (1)</td>
<td>32 (1)</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

* A total of 750 single cells are represented: 533 interferon protected cells and 217 control cells. The average control cell virus yields ranged from 55 to 285 p.f.u./cell for the various experiments, and the overall average was 161 p.f.u./cell.

† The figure given is the time of harvest. The virus was yielded during the 3 h immediately preceding that time.

§ All virus yields given for each harvest time, are expressed as percent control average and the number of single cells in each sample is given in parentheses, i.e., 80 (4) means the average virus yield size was 80 % of the control average for a sample size of 4.

§ These represent split yields in which virus was found at 2 succeeding harvest times. The time of burst was assumed to have occurred half way between the two times.

The rate at which virus is released is evidently not affected by interferon, since the burst occurs over nearly identical time periods: 17 min for treated cells and 15 min for the controls. In Fig. 4 the size of the virus yield, relative to the control average, is plotted against the time of yield. The largest bursts occur between 11 and 14 h and their size decreases thereafter.

Effect of interferon concentration on protection

Repeated sampling of virus yields from single, protected cells showed that increasing the concentration of interferon (a) decreased the size of the average yield (Fig. 5A), (b) decreased the number of yielding cells (Fig. 5A), and (c) increased the length of the virus latent period (Fig. 5B). Thus, there is a direct relationship between the concentration of interferon to which cells are exposed and the resultant antiviral effect.

Effect of multiplicity of infection on interferon protection

The effect of varying the input multiplicity of mengovirus on the above parameters was determined for control and interferon-treated cells (see Table 2). No differences in yield time, burst size or percent yielding cells was observed for control cells infected with 5, 10, or 20 p.f.u./cell. Since only a few of the protected cells infected at an input multiplicity of 5 or 10 p.f.u./cell yielded virus, comparisons of the average time and size of yield to the values found for an input multiplicity of 20 p.f.u./cell may not be valid. It is clear, however, that increasing the input multiplicity increased the proportion of protected cells which yielded virus. These results are in accord with the observations of Ho (1962) for vesicular stomatitis virus (VSV) and poliovirus RNA, and of Catalano & Baron (1970) for herpes simplex virus and encephalomyocarditis virus, but they differ from the observations of Hallum & Youngner (1966) for VSV virus and poliovirus RNA.
Fig. 4. Relation of burst size to time of burst. See Fig. 2 for experimental methods and Table 1 for a detailed presentation of the results. The average sizes of virus yields of interferon protected and control single cells are plotted for each harvest time which has a sample size of 5 or more cells. The control average was 161 p.f.u./cell. O--O, control; •--•, interferon protected cell.

Fig. 5A. Effect of interferon on the proportion of yielding single cells and on the time of virus yields. L cell monolayers were incubated with varying amounts of interferon for 11 h. The cells were dispensed into microdrops as described for Fig. 2A and 3. The time of virus yield was determined for each single cell and the average burst time (h post-infection) was determined for each concentration of interferon. Control yields averaged 127 p.f.u./cell, the average burst time for control cells was 13.5 h, and 93.5% of the controls yielded virus. O--O, % total; •--•, yield.

Fig. 5B. Effect of interferon on size of virus yield from producing cells. The average size of the virus yield from each yielding single cell (relative to control) was determined for each interferon concentration. Control yield/yielding cell was 134 p.f.u./cell.

Stability of the interferon-induced antiviral state

We propose that the major effect of interferon is to delay virus synthesis in protected cells. Two experiments show that this delay does not merely reflect a decay of the antiviral state.

Early removal of interferon from L cells

Monolayers incubated with interferon for 12 h and monolayers incubated with interferon for 3 h, and then without interferon for 9 additional h behaved identically after challenge with mengovirus.
Table 2. Effect of varying input multiplicities of mengovirus on interferon protection in single L-cells*

<table>
<thead>
<tr>
<th>No. of microdrops</th>
<th>Mengovirus input multiplicity</th>
<th>Units of interferon</th>
<th>Time of yield (h)</th>
<th>Size of yield (% control average)</th>
<th>Percent yielders (%)</th>
<th>Corrected percent yielders (%)</th>
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<tbody>
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<td>30</td>
<td>20</td>
<td>8</td>
<td>16.7</td>
<td>34</td>
<td>63</td>
<td>71</td>
</tr>
<tr>
<td>30</td>
<td>10</td>
<td>8</td>
<td>15.2</td>
<td>40</td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>30</td>
<td>5</td>
<td>8</td>
<td>17.0</td>
<td>45</td>
<td>17</td>
<td>17</td>
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<tr>
<td>18</td>
<td>20</td>
<td>0</td>
<td>12.2</td>
<td>103</td>
<td>89</td>
<td>—</td>
</tr>
<tr>
<td>18</td>
<td>10</td>
<td>0</td>
<td>12.2</td>
<td>97</td>
<td>100</td>
<td>—</td>
</tr>
<tr>
<td>18</td>
<td>5</td>
<td>0</td>
<td>12.2</td>
<td>101</td>
<td>100</td>
<td>—</td>
</tr>
</tbody>
</table>

* L cells were incubated with interferon, challenged with mengovirus, dispensed into microdrops, and virus yields were harvested as described for Fig. 3 A and 4. Control single cell virus yields averaged 277 p.f.u./cell.

Table 3. Effect of retaining interferon on single cells during infection*

<table>
<thead>
<tr>
<th>Experiment</th>
<th>No. of microdrops</th>
<th>Units of interferon</th>
<th>Input multiplicity</th>
<th>Average burst yield time (h)</th>
<th>Yielders (%)</th>
<th>Control yield size (%)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>Control</td>
<td>70</td>
<td>10.4</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>Control</td>
<td>19</td>
<td>11.2</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>30</td>
<td>4 units removed</td>
<td>70</td>
<td>12.7</td>
<td>77</td>
<td>82</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>4 units removed</td>
<td>19</td>
<td>15.4</td>
<td>35</td>
<td>13</td>
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<tr>
<td>1</td>
<td>30</td>
<td>4 units retained</td>
<td>70</td>
<td>12.1</td>
<td>93</td>
<td>71</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>4 units retained</td>
<td>19</td>
<td>12.3</td>
<td>45</td>
<td>25</td>
</tr>
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</table>

* L cell monolayers were incubated with either 0 units or 4 units of interferon for 11 h. The monolayers which had been incubated with 4 units of interferon were washed free of interferon at the time of virus challenge, split into two groups, and 4 units of interferon were added back to one of these groups with the challenge virus and maintained during the course of the experiment. Microdrops were set and harvested as described for Figs. 3 and 4. In two separate experiments, the cells were challenged with mengovirus at 70 p.f.u./cell and 19 p.f.u./cell and the average control yield sizes were 267 and 179, respectively.

Presence of interferon during infection

The data of Table 3 show that in experiments using two different multiplicities of mengovirus, it made little difference whether interferon was removed at the time of challenge (the usual procedure) or maintained throughout the experiment.

Thus, both types of experiment provide no evidence for either the decay of the antiviral state in the absence of interferon (at least for 9 h) or for the enhancement of the antiviral state in the continued presence of interferon beyond the normal 12 h incubation period.

DISCUSSION

In monolayers treated with interferon, the rate of virus synthesis was decreased and virus release was delayed relative to controls. This delay is believed to be a general effect of interferon since it has been demonstrated for a wide variety of virus-cell systems, and its cumulative effects over several cycles of replication have been implicated in the inhibition of plaque development on interferon treated monolayers.

The microdrop technique described permitted rapid isolation of any number of cells in small vol. of media (about 2 μl in a drop 2 mm in diam.). The manipulations involved for the isolation of single cells by this method required a minimal expenditure of time and did not significantly perturb the infectious cycle. It therefore was used to give a higher resolution to the study of the mode of action of interferon. At moderate levels of interferon,
the mengovirus yield from essentially every single L cell was delayed (Table 1). Thus, unlike
the results of Takemoto & Baron (1966) with encephalomyocarditis virus, there was no
evidence of the presence of a resistant fraction in a population of mengovirus. The delay of
virus release was striking; for example, while 5 units of interferon reduced the 10 h yield by
99%, the 24 h yield was reduced by only 60%. In both treated and control cells, virus was
released as a burst, i.e. a majority of the virus yield occurred during one harvest time. By
multiplying the percentage of split bursts (8-1% and 9-5% for control and protected cells,
respectively), by the length of the harvest interval (180 min) burst times of 15 and 17 min
were determined. This difference does not appear to be significant. Our data show that
whether interferon protects in an all-or-nothing or graduated manner depends on the time
the cells are sampled. At early times post-infection the few interferon treated cells which had
yielded, produced nearly normal amounts of virus, while at later times, the productive cells
yielded much less virus than the controls (Fig. 3).
Since interferon was routinely removed at the time of virus challenge in our experiments,
the possibility that the delayed production of mengovirus might reflect the decay of the
antiviral state was considered. Indeed, such a decay was reported by Baron et al. (1967) in
mouse cells challenged with VSV. Hallum, Thacore & Younger (1970) extended the studies
to chick cells and mouse cells challenged with either VSV or NDV and found that the rate at
which protection decayed was a function of both the virus and the cell. Two experiments were
performed to test for decay of protection in the mengovirus-L cell system. In one, interferon
was removed 9 h before challenge; and, in the other, interferon was left on the cells through-
out the experiment. In neither case were the results significantly altered (see Table 3).
Hence, although the antiviral state might decay, the rate of decay of protection against
mengovirus is slow and the change we observed in the latent period is independent of this.
Increasing the concentration of interferon (1) increased the latent period, (2) decreased the
number of yielding cells, and (3) decreased the size of the virus yield. Conversely, increasing
the multiplicity of challenge virus increased the number of yielding cells. These observa-
tions indicate that protection results when interferon, or a molecule induced by it, acts as a
competitive inhibitor of some cellular or virus product, such that the greater the interferon
concentration, the greater the number of antiviral molecules, the greater the degree of
competition, and the longer the delay in virus synthesis.
We propose that the direct effect of interferon is to delay virus synthesis. The observations
that both control and interferon protected cells show cytopathic effect by 10 to 12 h post-
infection, and that the size of the virus yield decreases with increased latent period, suggest
that this decrease is not due to the direct action of interferon, but rather to the state of the
cell. It is well documented that lysosomal hydrolases are activated as a consequence of
infection (Allison & Sandelin, 1963; Wolff & Bubel, 1964; Flanagan, 1965), and Bubel (1967)
showed leakage of protein from mengovirus infected guinea-pig spleen cells. The loss still
occurred when the synthesis of late virus proteins was blocked and was thus independent
of virus release. Therefore, control and interferon protected cells might well sustain increas-
ing degrees of cellular injury with time post-infection. The decreasing number of yielding
cells observed with increasing interferon concentrations, and the observation that no cells
yield virus after about 26 h, can then be explained by assuming that after 26 h the cell has
been damaged to the point where it can no longer support any virus production.
Thus, we suggest that the direct effect of interferon is to delay virus replication by means
of a competitive inhibition of some step in the cycle. Complete inhibition of virus production
is achieved when the initiation of synthesis is delayed beyond the point when a cell can still
support virus growth.
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REFERENCES


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