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Quantitative Aspects of Inhibition of Virus Replication by Interferon in Chick Embryo Cell Cultures

J. V. HALLUM AND J. S. YOUNGNER

Department of Microbiology, University of Pittsburgh, School of Medicine, Pittsburgh, Pennsylvania

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ABSTRACT

HALLUM, J. V. (University of Pittsburgh, Pittsburgh, Pa.), AND J. S. YOUNGNER. Quantitative aspects of inhibition of virus replication by interferon in chick embryo cell cultures. J. Bacteriol. **92**:1047–1050. 1966.—The effect of interferon on single cycles of replication of vesicular stomatitis virus and Mahoney poliovirus ribonucleic acid was studied in chick embryo cell cultures. The results showed that the titer of a given interferon preparation was independent of the input multiplicity of the challenge virus. In addition, the increase in virus yield with increasing virus challenge was a function of the number of infected cells, each of which yielded progeny at a level determined by the concentration of interferon to which the cells were exposed. These findings are not compatible with the concept that increases in the size of the virus challenge reverse or overcome protection of cells by interferon.

The inhibition of virus replication by interferon is commonly measured by two techniques, plaque reduction or yield inhibition. Plaque-reduction titers are calculated from the reciprocal of the interferon dilution which produces a decrease (usually 50%) in the number of plaques compared with untreated control cultures. It is difficult to study the dynamics of interferon action by the plaque-reduction method because of certain inherent complexities of the assay system. For example, the virus challenge dose is limited by the number of plaques which can be counted on each monolayer. Also, a diminution in the size of the plaques occurs as the number of plaques is decreased with increasing interferon concentration. Thus, when complete virus inhibition is apparent grossly, microscopic plaques may be present as a result of limited virus replication. The fact that plaque formation is the result of multiple cycles of virus replication makes the role of interferon difficult to interpret.

Yield-inhibition titers are calculated as the reciprocal of the interferon dilution which depresses the yield of progeny virus by some given percentage (usually 50%). The advantages of this assay system are that a wide range of multiplicities of infection can be used, and the effect of interferon on a single cycle of virus replication can be investigated. The chief disadvantage of yield inhibition is that a separate virus titration must be done with each sample harvested.

Both of the above techniques have been used to study the action of interferon (1, 4). It was concluded that the effect of interferon is not an "all or none" phenomenon, and that the inhibition produced is reversible by increasing the virus challenge. This suggests the possibility of a competitive equilibrium between interferon or an interferon product and the challenge virus at a site at which replication occurs. Further, this implies that the titer of an interferon sample is a function of the size of the virus challenge and, therefore, that the inhibition titer of a given interferon preparation would be lower when a higher concentration of challenge virus is used.

The present study was undertaken to investigate the effect of varying the concentration of the challenge virus on virus yield and on interferon titer by use of single cycles of replication of poliovirus ribonucleic acid (RNA) (2, 3) and vesicular stomatitis virus in chick embryo cell cultures.

MATERIALS AND METHODS

Interferon. Interferon was prepared in chick embryos by the method of Wagner (6) with the WS strain of influenza virus. Embryonated eggs (10 days old) were inoculated into the allantoic cavity with about 10^4 EID_{50} of virus. After incubation for 72 hr at 36 C, the allantoic fluids were harvested, pooled, and clarified by centrifugation at 520 $\times$ *g* for 20 min. The clarified interferon preparation was then centrifuged at 40,000 $\times$ *g* for 4 hr. The pellet, which contained

the bulk of the virus, was discarded, and the supernatant fluid was heated at 56 C for 1 hr to destroy residual infective virus. The interferon preparation then was concentrated to one-tenth its volume by dialysis against polyvinylpyrolidone. The titer of the interferon was determined by the plaque-reduction method, as previously described (7). The viral inhibitor in infected allantoic fluid was stable at 56 C, nonsedimentable at 100,000 $\times$ *g* for 2 hr, resistant to inactivation at *p*H 2 for 24 hr, trypsin-sensitive, effective in chick cells but not in mouse cells, and did not neutralize vesicular stomatitis virus (VSV), the test virus in the assay for interferon activity.

Chick embryo (CE) cell cultures. CE cell cultures were prepared from 10-day-old embryos. Minced tissues from decapitated embryos were washed, suspended in 0.25% trypsin, and stirred for 1.5 hr at room temperature. The dispersed cells were packed by centrifugation at 270 $\times$ *g* for 7 min, and then suspended in Eagle's medium containing 0.5% lactalbumin hydrolysate. The cells were further diluted to 600 times the packed-cell volume, and 4 ml of cell suspension was added to 60-mm petri dishes. The cultures were used after incubating at 37 C in a CO_2-gassed incubator for 48 hr. At this time, the cultures contained 2.1 $\times$ 10^6 to 2.4 $\times$ 10^6 cells per petri dish.

Infectious RNA was prepared from the Mahoney strain of type 1 poliovirus by extraction with cold phenol (5).

Yield-inhibition studies with infective RNA. CE cell cultures were treated with fourfold dilutions of interferon in serum-free Eagle's medium containing lactalbumin hydrolysate (0.5%). Control cultures were treated with medium only. The cultures were incubated at 37 C overnight, drained, and washed twice with 0.2 M $MgSO_4$ in physiological saline solution. Dilutions of infective poliovirus RNA were made in 2 M $MgSO_4$, and 0.2-ml volumes were added to three cultures. After a 20-min adsorption period at room temperature, the cultures were washed twice with serum-free medium, and incubated at 37 C with 3.0 ml of medium for 24 hr. The culture fluids then were harvested, pooled, and stored at -60 C until assayed for poliovirus infectivity by plaque formation in HeLa cell monolayers in petri dishes.

Yield-inhibition studies with VSV. Cultures of CE cells were inoculated with 3.0 ml each of fourfold dilutions of interferon in serum-free medium; control cultures received medium alone. After incubating overnight at 37 C, the cultures were drained, washed twice with 3.0 ml of phosphate-buffered saline (0.01 M, *p*H 7.2), and were challenged with dilutions of VSV in the same diluent. The challenge virus was added in 0.5-ml volumes to the cultures, and incubated at 37 C for 1 hr. Three cultures were used for each dilution of interferon. The inoculum was removed, and the cultures were washed twice with 3.0 ml of medium 199. A 3-ml amount of medium was then added, and the cultures were incubated at 37 C for 6 hr. This time was chosen as a result of a separate growth experiment done with VSV in primary CE cell cultures, which showed that a single cycle of replication was completed in about 6 hr. The supernatant fluids were then harvested, pooled according to virus challenge and interferon dilution, and stored at -60 C until assayed for VSV infectivity by plaque formation in CE cells.

RESULTS

Inhibition of replication of poliovirus RNA by interferon. The influence of the amount of poliovirus RNA used as challenge on the yield of infective poliovirus from cells treated with different dilutions of interferon was investigated, and the results obtained are summarized in Fig. 1 and 2. The plot of log yield of poliovirus against log of the concentration of the poliovirus RNA challenge (Fig. 1) shows that a given concentration of interferon reduces the yield of progeny virus by a fixed proportion, regardless of the size of the challenge dose. This was concluded from the observation that the lines obtained by plotting log yield against log challenge for all concentrations of interferon have slopes identical to the line obtained when no interferon is present.

The effect of interferon concentration on yield of poliovirus at four different challenge doses is shown in Fig. 2. It can be seen that the 50% inhibition end point is virtually identical for all challenge doses; the same is true of a 90, 99, or 99.9% end point. It was concluded from these results that the titer of an interferon sample is a function only of the concentration of interferon, and is independent of the challenge dose of RNA.

The data in Fig. 2 also point out that, when the interferon concentration was decreased 4-fold, the yield of progeny virus increased about 100-fold. This relationship was seen at all concentrations of RNA tested, and confirmed the observa-

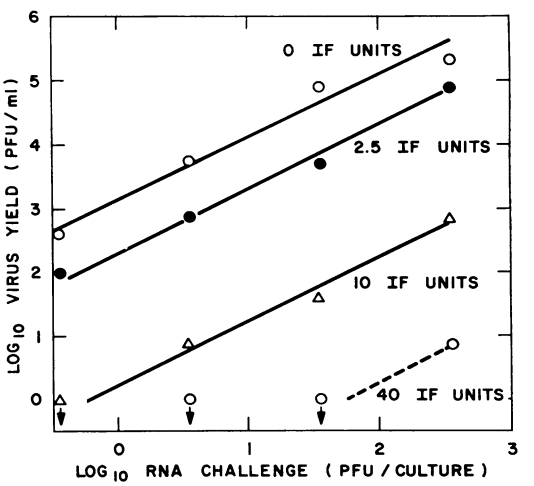

FIG. 1. *Effect of concentration of infective poliovirus RNA on poliovirus yield after pretreatment of CE cultures with different concentrations of interferon.*

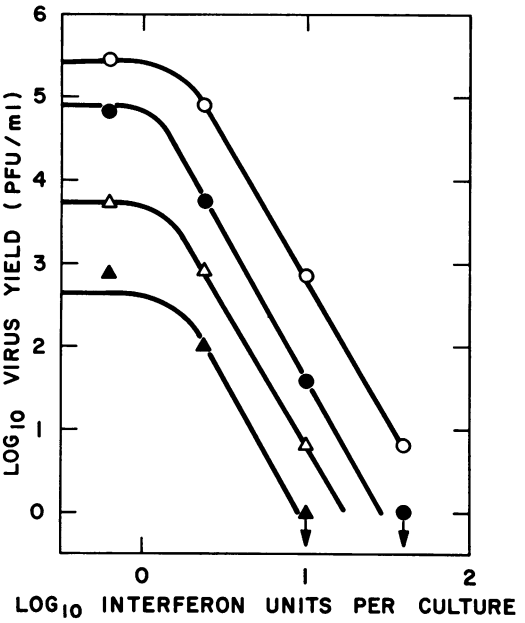

FIG. 2. *Effect of concentration of interferon used to pretreat CE cultures on yield of poliovirus at different levels of challenge by infective poliovirus RNA. Symbols: ○, 350 PFU per culture; ●, 35 PFU per culture; △, 3.5 PFU per culture; ▲, 3.5 × 10⁻¹ PFU per culture. The intercepts of the different curves represent the virus yields in cultures not treated with interferon.*

tions of others (1, 4) that the action of interferon is not an "all or none" phenomenon. To explain this graded response, it was necessary that all of the challenged cells be equally protected. If the presence of progeny virus were due to the infection of some unprotected cells which had received no interferon and thus were able to produce their maximal yield of progeny, decreasing the concentration of interferon fourfold would mean that the number of unprotected cells would increase fourfold. In this case, the number of progeny would be expected to be only fourfold greater than at the next higher concentration of interferon.

Inhibition of yield of VSV by interferon during a single cycle of replication. A serious limitation was encountered in the range of multiplicities of infection which could be tested conveniently by use of infective poliovirus RNA. Since the highest concentration of RNA employed involved the inoculation of 3.5×10^2 plaque-forming units (PFU) per 2.2×10^6 cells, the highest input multiplicity used was approximately 0.0002. To circumvent this limitation, the experiments described in the preceding section were repeated with an intact virus, VSV, with which higher input multiplicities could easily be obtained. CE cell cultures were incubated overnight with fourfold dilutions of interferon, and the cultures then were challenged with VSV by use of input multiplicities ranging from 12 to 1.2×10^{-6}. After 6 hr of incubation at 37 C, the fluids were harvested and the infective-virus yield was determined. The results of these experiments, which are summarized in Fig. 3 and 4, were similar to those obtained with poliovirus RNA.

Figure 3 shows that the lines obtained by a plot of the log of the virus yield against the log of the size of the virus challenge are parallel at all concentrations of interferon tested. This indicated that the proportionate decrease in yield of progeny virus brought about by a decrease in the the challenge virus is the same whether interferon is present or not, and is strong evidence that this proportionate decrease is due to a decrease in the number of cells infected by the challenge virus. The significance of these findings will be discussed later.

The effect of interferon concentration on yield of VSV at different challenge levels is shown in Fig. 4. As was the case with poliovirus RNA, the titer of an interferon preparation was independent of the challenge dose of virus. When compared with the yield in untreated control cultures, a given percentage of decrease in yield occurred at the same interferon concentration, regardless of the size of the virus challenge.

DISCUSSION

When cells are treated with a given concentration of interferon, they develop a certain level of

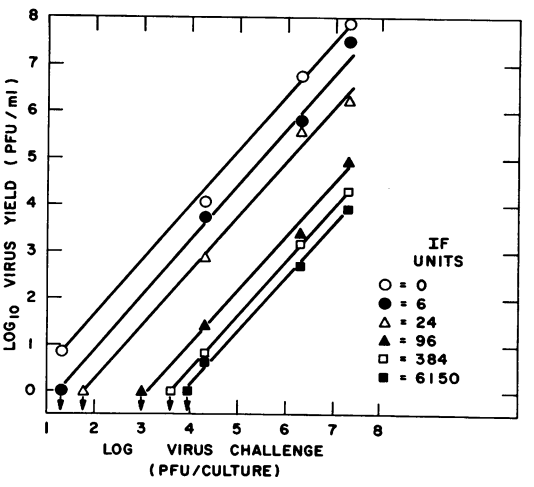

FIG. 3. *Effect of concentration of challenge virus on yield of VSV after pretreatment of CE cultures with different interferon concentrations.*

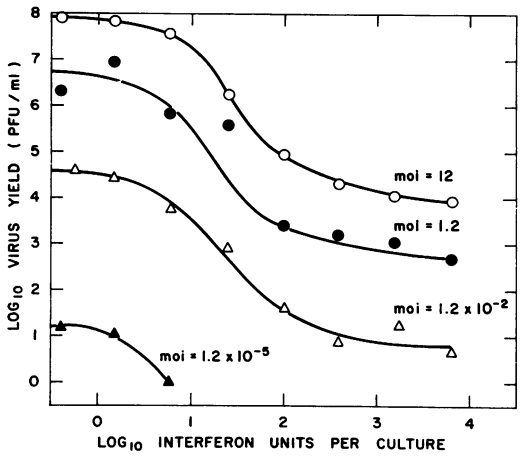

FIG. 4. *Effect of interferon concentration used to pretreat CE cultures on virus yield at different levels of challenge with VSV. The intercepts of the different curves represent the virus yields in cultures not treated with interferon.*

protection against virus challenge. The level of protection is expressed as a reduction in the virus yield, as compared with untreated control cells, and is proportional to the concentration of interferon to which the cells were exposed. With effective doses of interferon, all of the cells in the culture are equally protected. This implies that the virus yield is not due to the presence of a small fraction of unprotected cells which are producing a maximal yield of progeny. For example, in the experiments with poliovirus RNA, the data (Fig. 2) show that a 4-fold decrease in interferon concentration caused a 100-fold increase in poliovirus yield. If the progeny had come from completely unprotected cells, the increase in virus yield also would have been fourfold. These findings are interpreted to mean that the level of production of progeny virus in each cell is determined by the concentration of interferon, but the number of progeny-producing cells is dependent upon the input multiplicity of the challenge virus. These interpretations also explain why it was found that the titer of an interferon sample is independent of the size of the virus challenge.

If the dependence of virus yield on the size of the virus challenge is studied at a single interferon concentration, it appears as though the effect of increasing the virus challenge is to reverse or overcome the protection conferred on the cells by interferon. However, this is misleading. The experiments described show that reversal or overcoming of interferon protection is unlikely. As already stated, increases in virus yield resulting from higher virus challenge are due to increases in the number of progeny-producing cells. These cells are all protected by interferon and yield virus at a level governed by the concentration of interferon to which they were exposed.

ACKNOWLEDGMENTS

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ADDENDUM IN PROOF

Since this paper was submitted for publication, work by R. Z. Lockart, Jr., and T. Sreevalsan (in *Viruses, Nucleic Acids and Cancer*, p. 447–461, 1963, The Williams & Wilkins Co., Baltimore) bearing on the lack of reversibility of interferon action has come to our attention.

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