INTERACTION OF AN INTERFERON WITH L CELLS

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ABSTRACT

LOCKART, ROYCE Z., JR. (The University of Texas, Austin) AND BARBARA HORN. Interaction of an interferon with L cells. J. Bacteriol. **85**:996–1002. 1963.—Data were presented on the effect of time of exposure and concentration of an interferon in provoking viral inhibition in L cells. Populations of L cells which made reduced amounts of Western equine encephalomyelitis virus as a result of treatment with interferon did so at reduced rates proportional to the concentration of interferon used. Virus yields were maximal, however, 25 hr after challenge regardless of the amount of virus produced. Such populations of cells contained a proportion of cells no longer able to produce infective virus, while the average maximal yield of the remainder of the cell population was reduced. It was suggested that only cells which made new virus underwent cytopathic effects. The rate of viral inhibition in monolayers of L cells was dependent on the concentration of interferon added, but inhibition was nearly maximal at 8 hr, regardless of the interferon concentration. Viral inhibition was shown to persist in multiplying cells, but it gradually diminished. The amount of inhibition after either one or two cell divisions was greater in those cultures treated with greater amounts of interferon. Viral inhibition could be passed through cell division with no loss when cells were incubated with a sufficient concentration of interferon. A model of interferon action based on the preceding data was presented.

An interferon is produced as a result of the infection of strain L mouse cells with Western equine encephalomyelitis virus (WEE; Lockart, 1963). Treatment of cells overnight with a sufficient concentration of interferon produced cultures of cells which failed to show cytopathic effects (CPE) and which were inhibited in their ability to produce virus. Varying degrees of CPE and viral inhibition were noticed when lesser doses of interferon were used, but they were not well correlated. The experiments to be described were designed to study the quantitative aspects of the interaction of an interferon and strain L mouse cells. Ho (1962*a*), in his excellent review, pointed out the scarcity of studies of interferon action in continuous cell lines. Although he (1962*b*) reported on some kinetic relationships of interferon with primary chick embryo cells, we know of no such studies with serially cultivatable cells. Therefore, we have attempted to understand the relationship of the interferon dose to the resulting state of viral inhibition and lack of CPE in such cells.

MATERIALS AND METHODS

Strain L 929 cells were routinely grown as stationary cultures in Eagle's (1955) medium supplemented with 5% horse serum. All experiments were initiated on monolayer cultures of L cells in 60-mm petri dishes. Monolayers were prepared in replicate by the addition of 8×10^5 cells from a trypsinized suspension of cells to 60-mm petri dishes in 5 ml of medium. At the time of use, 17 to 24 hr later, resulting monolayers contained between 1 and 1.5 million cells.

Monolayers of primary chick embryo tissues were prepared according to the methods described by Dulbecco and Vogt (1954).

Stock suspensions of a strain of WEE capable of causing complete CPE on L-cell monolayers were prepared on chick embryo tissues. Virus suspensions were distributed in 1-ml quantities and kept at −30 C until used. Infective titers were determined by the plaque-assay method on monolayers of chick embryo tissues (Lockart, 1963). Duplicate samples were plated for each determination.

Suspensions of interferon were prepared by harvesting the fluids from L-cell cultures containing about 30 million cells which had been infected 48 hr previously with WEE. The fluids were centrifuged at $1,200 \times g$ for 20 min to remove cellular debris. Some suspensions received no further treatment. Some were centrifuged again in a

Spinco model L centrifuge to reduce the amount of active virus, or exposed to an ultraviolet germicidal lamp for 5 min at a distance of 15 cm to inactivate the virus present.

To assay for interferon activity, interferon suspensions were diluted serially in twofold steps in medium containing 5% horse anti-WEE serum. Samples (2 ml) were added to duplicate monolayers of L cells that had been prepared 17 to 24 hr previously, as already described. It was found that prior removal or inactivation of virus did not influence the action of the interferon present when added in this manner (Lockart, 1963). Cell monolayers containing the serial dilutions of interferon were then incubated at 37 C for 17 to 24 hr or for the length of time specifically noted. After incubation, the medium was removed and the monolayers were washed two times with PBS (Dulbecco and Vogt, 1954) to which was added bovine serum albumin fraction 5 (BSA) to a concentration of 0.1% (PBS + BSA; BSA is a product of Mann Research Laboratories, New York, N.Y.) prior to the addition of virus. WEE at a multiplicity of 10 or greater was added in 0.5 ml of PBS + BSA and allowed to adsorb for 1 hr at 36 C. Unadsorbed virus was removed by three successive washes with PBS + BSA, after which 5.0 ml of Eagle's medium containing 5% horse serum were added.

The proportion of virus-producing cells (infectious centers) was determined in the following way. Cultures were treated with interferon and challenged with virus as described above. After three washes with PBS + BSA to remove unattached virus, the cultures were washed once rapidly with 2 ml of warm 0.05% trypsin; 2 ml of warm trypsin were then added and, during a further incubation at 37 C for 5 min, the cells became detached. They were added to 8.0 ml of cold medium, then collected by centrifugation. The medium was removed and replaced with 1.0 ml of medium containing 10% antiserum. This mixture was incubated at room temperature for 45 min with mixing every 2 to 3 min. The cells were washed again with 10 ml of medium, resuspended in 10 ml of medium, and counted in a hemocytometer. The cell suspension was diluted in cold medium, and 0.5-ml samples were added to three or four previously washed chick embryo monolayers; 1 ml of nutrient overlay agar was added immediately, and an additional 9.0 ml when the initial layer had hardened. Cells were removed from the suspending medium by centrifugation at 1,000 × *g* for 10 min, and the medium was assayed for free virus in the above manner. It was never necessary to make corrections for free virus. The whole experiment was concluded within about 4 hr after the addition of virus, and before the end of the latent period of WEE in L cells.

RESULTS

Kinetics of virus production in interferon-treated cultures. In most experiments, varying dilutions of interferon were incubated overnight with replicate cultures of cells. The cultures were then challenged by the addition of a virus multiplicity adequate to insure infection of the whole culture. The resulting CPE was estimated, and maximal virus production measured. To determine the time of maximal virus production for a given culture, one-step growth curves were performed on cultures of cells treated overnight with varying doses of interferon. A representative experiment is shown in Fig. 1. Those cultures in which the amount of virus produced was less than in the controls showed a longer latent period and a reduced rate of virus production. These findings are consistent with the idea that all the cells, even those which do produce virus, are affected by their prior treatment with interferon and that virus production is not an all-or-none phenomenon. Cultures, nevertheless, produced maximal virus titers by 25 hr, and the titers remained relatively constant until 31 hr. Between 25 and 30 hr, no significant amount of virus remained attached to the cells such that it could be released by freezing and thawing. In the remainder of the experiments to be described, samples for estimation of virus production were removed between 25 and 30 hr, and were considered to represent maximal yields.

Reduction of virus yield and yielding cells by interferon. The reduction in the amount of virus produced could be a result of a decreased yield of virus by all the cells (Levine, 1962), a reduced number of virus-yielding cells (Cooper and Bellett, 1959), or a combination of both (Ho, 1962*b*). To decide which was the case in the present system, a comparison was made between the 25-hr virus yields and the proportion of yielders in cultures treated overnight with varying doses of interferon. The results are shown in Fig. 2 and tabulated in Table 1. In Fig. 2, the per cent of virus yields and infectious centers relative to controls is

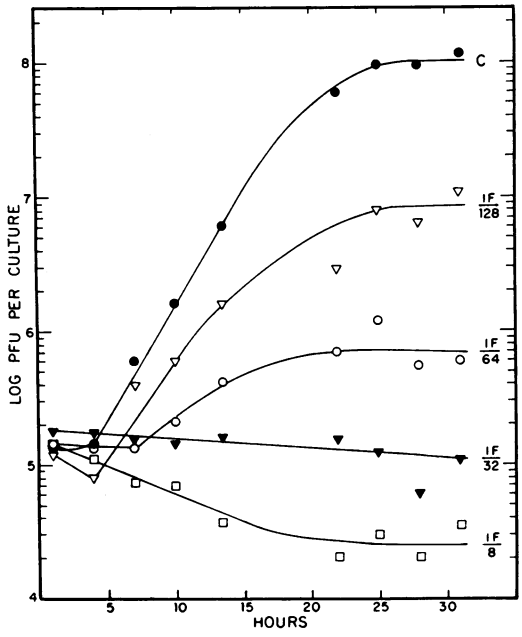

FIG. 1. *Growth curve to determine time of maximal virus production. Replicate monolayers were incubated overnight with varying dilutions of interferon or medium. Medium with or without interferon was removed, and cultures were washed two times, then challenged by the addition of 10 or more PFU per cell of Western equine encephalomyelitis virus. After 1 hr for adsorption, free virus was removed by three washes. Samples were removed at the indicated times and frozen until assayed for virus content. A volume of medium equal to that removed was added to the cultures after each sampling.*

plotted semilogarithmically against the dilution of interferon added. Control cultures which received no interferon and which should have contained only infected cells showed only about 32% infectious centers. This was considered to represent the efficiency of the assay for infectious centers, and all infective-center data were corrected by multiplying the obtained values by 1/efficiency of assay. A certain proportion of the cells treated with interferon did, indeed, become nonvirus yielders. The reduction of virus yield at each dose of interferon, however, greatly exceeded the reduction in the number of infective centers. The remaining virus-producing cells synthesized an average amount of virus, which became less at each increasing dose of interferon. At a sufficiently high dose of interferon, the average production per cell would be expected to approach a minimal level below which a cell would no longer be assayed as an infectious center. Any decrease in virus yield after this would then also be reflected as a decrease in the number of virus yielders. In compliance with the above expectation, the reduction in virus yield and virus-producing cells became parallel in cultures treated with an interferon suspension diluted 1:16 and 1:8 (Fig. 2). The calculated yields per virus-producing cell (corrected for the efficiency of infectious-center assay) at increasing doses of interferon are shown in Table 1. The data (Table 1) also illustrate the lack of correlation between the amount of CPE and the over-all virus yields. More CPE is always found than might be expected by determining the amount of virus produced relative to control cultures. Although not exact, there is, on the other hand, a fairly good correlation between the amount of CPE and corrected proportion of the population that produces virus. It seems reasonable to suggest that only cells making virus are destroyed, even though they make an amount of virus reduced below that of untreated cells. It is clear that a single unit of interferon does not necessarily make a cell a nonvirus yielder nor protect it from CPE, but a sufficient dose of interferon may do both.

Effect of dose on onset of interference. The above data suggest that, to obtain a given level of virus inhibition, a culture must take up a certain amount of interferon. If this is so, the time necessary to reach a given level of virus inhibition should depend on the interferon concentration. This is demonstrated in Fig. 3, which shows the amount of virus inhibition that ensued at varying times after the addition of interferon. The time taken to reach 98% virus inhibition was 1.2, 2.2, and 6.8 hr, respectively, at 1:2, 1:16, and 1:64 dilutions of interferon. It is also clear that interferon uptake was almost complete by 12 hr. Protection against cytopathic effects, like virus inhibition, was also dependent on the uptake of enough interferon. In the cases shown, interferon uptake causing 99.95% virus inhibition was necessary to prevent the appearance of any detectable cytopathic effects in the cultures, and the time necessary for this effect was dose-dependent. These data again suggest that absence of cytopathic effects in a cell culture results only when the proportion of nonvirus-yielding cells is sufficiently great to prevent CPE or to mask the few cells that do undergo degeneration.

Duration of interference as a function of interferon dose. Interference is only a transient state.

TABLE 1. *Effect of varying doses of interferon on the proportion of virus-yielding cells and virus yields*

Expt no.	Interferon dilution	CPE[a] at 72 hr	Virus yield (PFU/culture)[b]	Per cent yield	Cells plated	Infectious centers[c]	Per cent infectious centers	Yielders per culture[d]	PFU/yielder
1	None	++++	4×10^7	100	80	26.6	33.2	1.8×10^6	22
2	None	++++	2.9×10^7	100	60	19.0	31.7	1.4×10^6	21
1	1:128	+++	1.8×10^7	45	ND	ND	ND	ND	ND
1	1:64	++	1.1×10^7	27	170	29.0	17	9.3×10^5	12
2	1:64	++	4.9×10^6	17	110	24.0	21.8	9.8×10^5	5
1	1:32	+	1.7×10^6	4.6	180	20.0	11	4.8×10^5	3.6
2	1:32	+	1.5×10^6	5.0	139	10.0	7.2	3.2×10^5	4.7
1	1:16	−	6.5×10^5	1.6	1,000	55.6	5.6	3.0×10^5	2.2
2	1:16	±	4.1×10^5	1.4	595	26.6	4.5	2.0×10^5	2.1
1	1:8	−	3.1×10^5	0.75	1,000	23.6	2.4	1.3×10^5	2.4
2	1:8	−	1.9×10^5	0.64	785	10.6	1.3	5.7×10^4	3.3
2	1:2	−	4.0×10^4	0.14	8,100	56.3	0.7	3.1×10^4	1.3

[a] CPE = cytopathic effects: − = none, ± = to 5%, + = 5 to 25%, ++ = 25 to 75%, +++ = 75% to 95%, ++++ = complete.

[b] Monolayers contained 1.8×10^6 cells in experiment 1 and 1.4×10^6 in experiment 2 at the time of challenge.

[c] The number of infectious centers represents the average number of plaques on three chick embryo monolayers.

[d] The average number of yielders per culture was calculated by assuming that the control cells were all infected and the efficiency of infective-center assay was 0.33 and 0.32, respectively. The observed per cent of infective centers was multiplied by 1/efficiency assay, and this times the total cells per culture gave the number of yielders per culture corrected for efficiency of infective-center assay.

Cells rendered resistant to the effects of a challenge virus revert back to susceptibility upon subsequent growth.

Data, so far presented, suggest that a given dose of interferon causes cells to make a reduced amount of virus and that larger doses render them nonvirus producers. The latter condition has been suggested as responsible for the absence of CPE. From these findings, one might predict that the loss of the inhibited state should proceed in reverse order, i.e., complete CPE with reduced virus yields prior to full virus yields. The time necessary for this reversion should be dependent on the quantity of interferon taken up by the culture. To test the above predictions, the following experiments were performed.

Cultures were allowed to reach a given state of interference by allowing them to incubate overnight with interferon. Interferon was then removed, the cultures were washed, and 5.0 ml of regular medium were added to each. At the time of interferon removal and at several succeeding 24-hr intervals, duplicate cultures were challenged as before, with a high multiplicity of WEE. Replicate cultures were counted each time. The amount of virus present 25 hr after challenge and the amount of CPE at 72 hr were determined. The above predictions were substantiated (Table 2). Those cultures treated overnight with sufficient interferon to render them almost resistant to CPE (1:64 in experiment 1, Table 2) reduced the virus yield by 99.34%. All resistance to CPE was gone 24 hr later, but they still showed the effects of interferon action, as they produced only 6.25% as much virus as control cultures. After a further 24-hr period of incubation in the absence of interferon, replicate cultures produced about 28% as much virus as controls. It was not possible to carry out these experiments for more than 3 days due to overgrowth of the cultures. Even at 3 days, there was

a marked decrease in the virus production in control cultures.

An interesting point from these experiments was that both inhibition of virus production and protection against CPE were passed to daughter cells. As the data in Table 2 indicate, cultures of cells treated overnight with a 1:4 dilution of interferon multiplied twofold in the 24-hr period following the removal of the interferon. One might have expected at that time a population of cells, half of which would be completely susceptible. However, such cultures did not produce more virus than did replicate cultures 24 hr previously, nor did they show any CPE. No interfering activity could be found in the fluids removed at the time of challenge or in the fluids of cultures that had been disrupted by three cycles of alternate freezing in a Dry Ice-alcohol mixture and thawing. Further, the number of cell generations

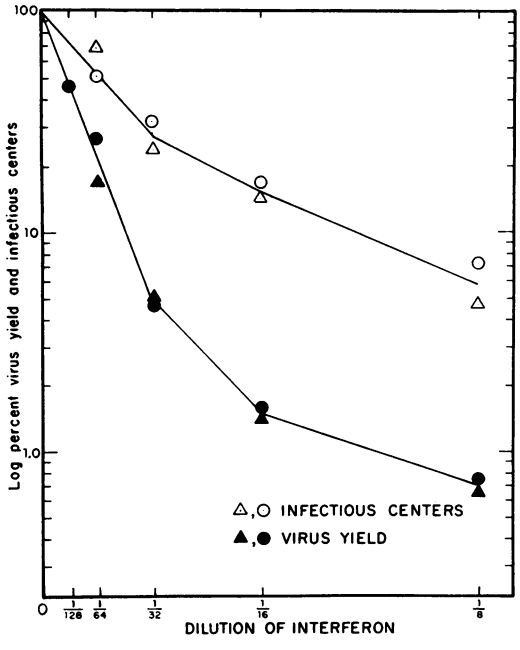

FIG. 2. *Per cent of virus yields and infectious centers of interferon-treated cells. Replicate cell monolayers were incubated overnight with varying dilutions of interferon or medium. The medium was removed, and the plates were washed two times. They were challenged by the addition of 10 or more PFU per cell of Western equine encephalomyelitis virus, allowed 1 hr for virus adsorption, then washed three times more. Infectious centers were determined as described in the text. Samples were removed 25 hr after challenge and frozen until assayed for virus content.*

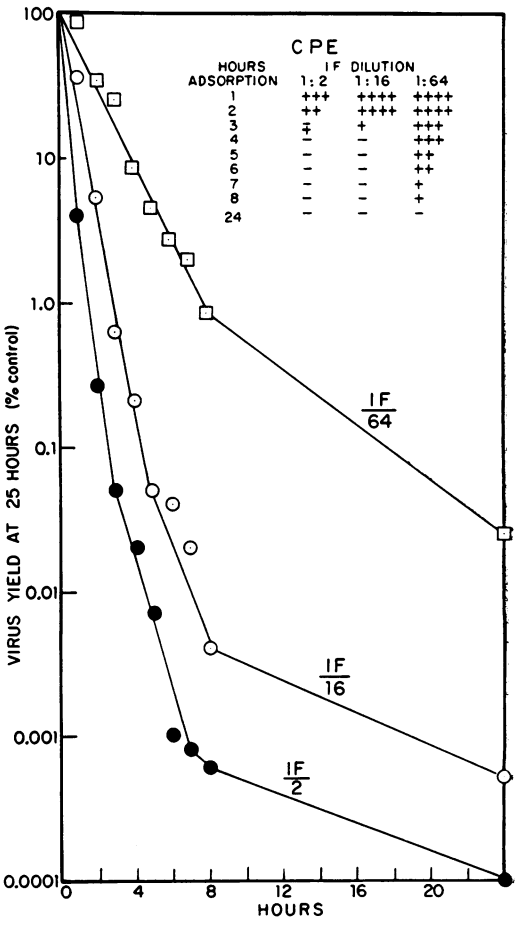

FIG. 3. *Amount of virus inhibition at varying times after addition of interferon. Replicate monolayers of cells were washed, and 2.0 ml of varying dilutions of interferon were added. Each hour for 8 hr, and at 24 hr after the addition of interferon, duplicate plates were removed, washed three times to remove excess interferon, and challenged by adding 10 or more PFU per cell of Western equine encephalomyelitis virus. Samples were removed 25 hr after challenge and frozen until assayed for virus content. Control monolayers were challenged initially, at 8 hr and at 24 hr. Cytopathic effects were estimated 72 hr after challenge and are indicated as follows: − = none, ± = 0 to 5%, + = 5 to 25%, ++ = 25 to 75%, +++ = 75 to 95%, ++++ = complete destruction.*

through which the inhibition can be passed appears to be dependent on the dose of interferon which the cultures originally receive.

These results suggest that interferons or interferon products are bound in some manner to cellu-

TABLE 2. *Duration of interference in cells treated with interferon*

Expt no.	Interferon added (dilution)	Time of challenge (hr after removing interferon)	Avg. no. of cells per monolayer ($\times 10^6$)	Virus yield (PFU/cell)	Virus yield (% control)	CPE* (at 72 hr)
1	None	0	1.1	1,220	100	++++
	None	24	2.0	625	100	++++
	None	48	3.9	370	100	++++
	1:4	0	1.1	0.14	0.011	−
	1:4	24	2.3	0.16	0.025	−
	1:4	48	3.1	2.9	0.78	+
	1:16	0	1.0	0.23	0.019	−
	1:16	24	2.2	5.0	0.8	++
	1:16	48	3.2	29.0	7.8	++++
	1:64	0	1.0	8.0	0.66	+
	1:64	24	2.3	39.0	6.25	++++
	1:64	48	3.9	103.0	27.8	++++
2	None	0	1.1	226	100	++++
	None	24	2.3	239	100	++++
	None	48	4.6	42	100	++++
	1:4	0	1.1	0.12	0.05	−
	1:4	24	2.2	0.12	0.05	−
	1:4	48	3.3	0.32	0.75	+
	1:16	0	1.2	0.12	0.05	−
	1:16	24	1.8	0.67	0.28	+
	1:16	48	3.6	6.3	14.9	++
	1:64	0	1.1	0.17	0.07	−
	1:64	24	2.2	9.0	3.8	++
	1:64	48	4.4	17.0	16.6	++++

* See footnote to Table 1.

lar structures. They are distributed at cell division or lost as a result of cellular metabolism.

Discussion

Our conclusion that interference induced by interferon is not an "all-or-none" phenomenon agrees with the conclusion reached by Ho (1962*b*) in chick embryo cells. It may also serve to explain why Levine (1962) found no significant reduction in virus-yielding cells in chick embryo cells treated with an interferon from Newcastle disease virus, although he found a significant reduction in the virus yield. It is probable that he employed insufficiently concentrated interferon or did not allow sufficient time for interferon uptake to permit a significant reduction in the number of cells able to produce virus. On the other hand, there are not enough data on the kinetics of cell-interferon interaction in different systems to warrant the generalization that all interferons act in the same manner.

It was further shown that there existed a rather good correlation between the proportion of cells calculated to be virus yielders and the amount of CPE observed. Conversely, there did not appear to be a correlation between the maximal virus yields of a culture and the amount of resulting CPE. Less virus than might be expected on the basis of the amount of CPE was invariably found in interferon-treated cultures. This is easily explained on the basis that the average yield per virus-producing cell was reduced. Absence of CPE in a cell would result if the cell became a non-yielder.

The transfer of the inhibited state through a doubling of the population in the absence of any demonstrable interferon suggests that as interferon is taken up by the cells it is bound irreversibly or disappears as such, and causes the production of some bound substance capable of surviving cell division while still producing its virus-inhibitory effect. Although the amount of virus inhibition was usually diluted upon growth, pretreatment with a sufficiently concentrated dose of interferon permitted doubling of the cell population with no loss of inhibitory activity. These data, coupled with the finding that virus production occurred more slowly in interferon-treated cultures than it did in controls (*see* Wagner, 1961), lead us to propose the following scheme of interferon action.

Interferon is taken into the cell where it acts by binding to critical sites for virus production. Virus synthesis in such cells is carried out by the remaining sites. The result is a reduced amount of virus produced at a reduced rate. Binding of interferon to some critical proportion of the sites prevents the cell from producing any virus. Such cells are protected from destruction. In the absence of interferon in the environment, the cells multiply, and the number of sites retaining interferon is reduced by dilution. If sufficient interferon were taken up by the cells initially, more than a single generation might be required before the number of unbound sites falls below the critical number for complete inhibition. Once this occurs, however, the cells again make partial yields and are subsequently destroyed. Work with chick embryo cells suggests that the sites might be involved in viral ribonucleic acid synthesis (Lockart, Sreevalsan, and Horn, 1962), but this has not yet been checked in L cells.

ACKNOWLEDGMENTS

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