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Factors Affecting the Sensitivity of Different Viruses to Interferon

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When the sensitivities to interferon of Newcastle disease virus (NDV) and vesicular stomatitis virus (VSV) were compared by the plaque reduction method in chick embryo cell cultures, NDV was found to be 45-fold more resistant than VSV. This difference was exaggerated when a multiple-cycle yield inhibition method was employed. In marked contrast, when the same viruses were tested by a single-cycle yield inhibition method, the difference in sensitivity to interferon of the two viruses was virtually eliminated. Further investigation showed that, in chick embryo cells exposed to interferon, the resistance to NDV decayed more rapidly than resistance to VSV. This finding explained the divergent results obtained with the two viruses when single- or multiple-cycle replication techniques were employed. Experiments carried out with L cells showed that cellular antiviral resistance decayed much more slowly in these cells than in chick embryo cells. Consequently, when measured by the plaque reduction method in L cells, no difference was observed in the sensitivity to interferon of VSV and NDV_{pi}, a mutant of NDV which replicates efficiently in L cells. A procedure is suggested for determining the relative sensitivities to interferon of different viruses under conditions which minimize the role of decay of antiviral resistance in the host cells.

Wide variations in the response of different viruses to inhibition by interferon (3, 9–11, 13, 17) have suggested a ranking of viruses based on their sensitivity to this inhibitor (15). The arbovirus group is usually considered most sensitive; vesicular stomatitis virus (VSV), vaccinia, and other poxviruses are of intermediate sensitivity; and Newcastle disease virus (NDV), herpes simplex, and cytomegalovirus (9) are the least sensitive. An example of the basis for this ranking is the difference in sensitivity of VSV and NDV to interferon when tested by a plaque reduction method in chick embryo (CE) cell cultures; a concentration of interferon which completely inhibits plaque formation by VSV has little or no effect on NDV. Any mechanism which is invoked to explain the action of interferon must take into account such variations in the susceptibility of different viruses. The experiments reported in this paper were designed to answer the following questions. (i) Is the variation in sensitivity of viruses to interferon in a given host cell a function of the method of assay? (ii) What determines the sensitivity of a given virus when tested in different host cells by the same method of assay?

MATERIALS AND METHODS

Cell cultures. Mouse L cells (clone 929) and primary CE cell cultures were propagated and maintained in Eagle's minimal essential medium plus 4% calf serum as described in detail elsewhere (18).

Viruses. The Herts strain of Newcastle disease virus (NDV_o) and a small plaque mutant (NDV_{pi}, clone 2) isolated from L cells persistently infected with NDV_o were used in this study. The viruses were grown in CE cell cultures and in chick embryos as previously described (14). Both NDV_o and NDV_{pi} replicate in CE cell cultures without the production of detectable amounts of interferon. Infection of L cells with NDV_o results in an abortive infection which yields few infective virus particles. In contrast, NDV_{pi} replicates efficiently in L cells and produces infective virus. Both NDV_o and NDV_{pi} induce interferon synthesis in L cells (14).

The Indiana strain of VSV was propagated in L cells as described earlier (18).

Preparation of interferon. L cell interferon was prepared as follows: L cells grown in 32-oz bottles (ca. 900 ml) were infected with NDV_o at an input multiplicity of infection (MOI) of 100 plaque-forming units (PFU) per cell. After 60 min of adsorption at 37 C, the cells were washed, refed with medium, and incubated at 37 C for 48 hr. The culture fluids were harvested and clarified by centrifugation, and the infectivity was inactivated by treatment at *p*H 2 for 2 weeks. Interferon preparations were stored at −20 C.

Chicken interferon was prepared in embryonated eggs by the method of Wagner (16) with the WS strain of influenza virus; details of the procedures have been reported earlier (4).

Interferon assay. Plaque reduction assays were

carried out in L or CE cell cultures as described previously (18).

Two types of yield reduction methods were utilized. (i) Single-cycle yield reduction was determined as follows. Cell cultures were exposed to serial dilutions of interferon for 20 hr at 37 C. The cells were washed twice with growth medium and infected with the appropriate virus at an input MOI of 1. After adsorption for 60 min at 37 C, the cultures were washed twice to remove unadsorbed virus, refed with medium, and reincubated at 37 C. Fluids from infected cultures were harvested after the time required for a single cycle of replication. In CE cell cultures this time was 6 hr for VSV and 10 hr for NDV_o and NDV_{pi}. The per cent inhibition of yield by interferon was calculated by comparing the virus yield from cultures treated with interferon to the yield from untreated control cultures. (ii) Multiple-cycle yield reduction was determined in a similar manner except that the fluids from infected cells were harvested 24 hr after infection, at which time the cells were completely destroyed.

RESULTS

Inhibition of NDV and VSV by interferon in CE cell cultures: plaque reduction method. An experiment was carried out to confirm that NDV is more resistant to inhibition than VSV when tested by the plaque reduction method in CE cell cultures. Serial 0.5 $\log_{10}$ dilutions of chick interferon were added to CE cell cultures; after 20 hr at 37 C, the interferon was removed, the cells were washed, and the cultures were divided into three groups. Each group was challenged with 40 to 60 PFU of either NDV_o, NDV_{pi}, or VSV. The per cent reduction of plaques at each interferon dilution was calculated from the number of plaques of each virus in control cultures which had been incubated with medium only. The relative sensitivities of the three viruses to inhibition by interferon are shown in Fig. 1. The inhibition of VSV plaques was used to calculate the number of units of interferon present in each dilution, as shown in the abscissa of Fig. 1; one unit of interferon was the amount required to inhibit VSV plaque formation by 50%. The highest concentration of interferon (70 units) used in this experiment produced only a 40% inhibition of plaque formation by the wild type NDV_o. In the case of the mutant NDV_{pi}, 15 units of interferon was required to produce a 50% plaque reduction. It is interesting that by extrapolating from the inhibition data plotted in Fig. 1, the titer of the chick interferon would be 15 units, 45 units, and 700 units per 3 ml if NDV_o, NDV_{pi}, or VSV, respectively, was used as the challenge virus. These data amply support the claim that NDV is relatively more resistant than VSV to inhibition by interferon when tested by the plaque reduction method in CE cell cultures. Additional experiments were carried out in CE cell cultures by using yield reduction methods in order to determine the influence of single and multiple replication cycles on the relative sensitivities of different viruses to chick interferon.

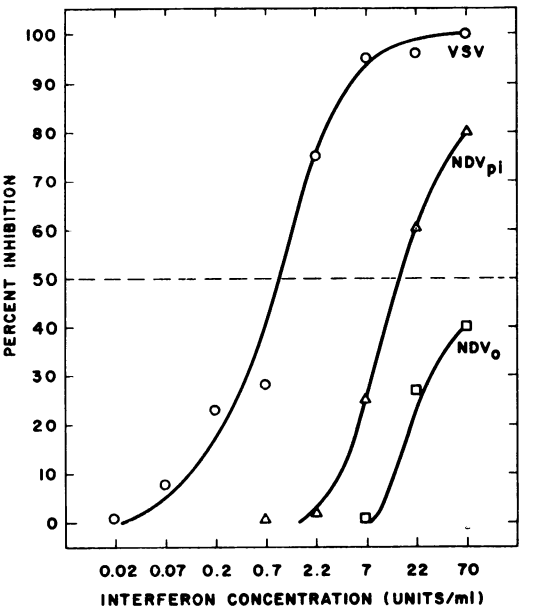

FIG. 1. *Inhibition of NDV and VSV by interferon in CE cell cultures: plaque reduction method. Cell cultures were incubated with 0.5 $\log_{10}$ dilutions of interferon for 20 hr at 37 C, washed, and challenged with 40 to 60 PFU of NDV_o (□), NDV_{pi} (△), or VSV (○). Cultures incubated with medium alone were used as control. The units of interferon shown on the abscissa were calculated from the amount required to inhibit VSV plaque formation by 50% (one unit).*

Inhibition of NDV and VSV by interferon in CE cell cultures: yield reduction methods. CE cell cultures were incubated overnight with interferon dilutions and infected with either NDV_o, NDV_{pi}, or VSV at an input MOI of 1 as described above. After 24 hr of incubation, when complete destruction of the cells had occurred, the culture fluids were harvested and assayed for infectivity in CE cell monolayers. Virus yield was also measured in control cultures exposed for 24 hr to medium only. A plaque reduction assay with the three viruses was carried out simultaneously in interferon-treated and control cultures to provide a standard for comparison and to calculate (from the VSV data) the number of interferon units in each dilution used to treat the cells. The results plotted in Fig. 2 show that NDV_o, NDV_{pi}, and VSV were less sensitive to interferon when tested by the multiple-cycle yield reduction method than when tested by the plaque reduction technique, although the three viruses maintained the same relative sensitivity to interferon. In the case of NDV_o, 500 units of interferon (as determined by VSV plaque reduction) produced a 35% decrease

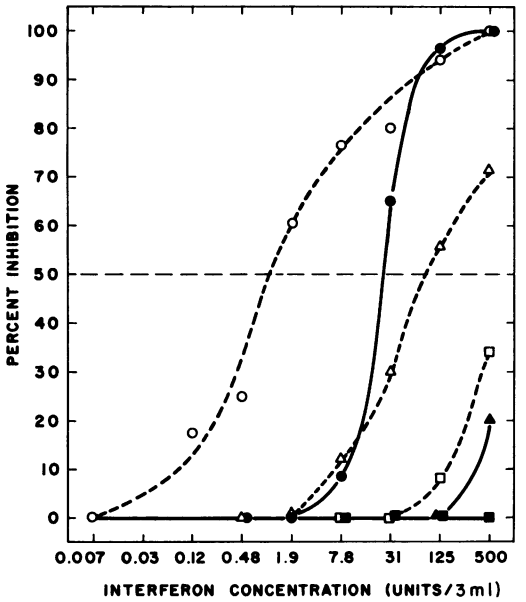

FIG. 2. *Inhibition of NDV by interferon in CE cell cultures: multiple-cycle yield method. Cell cultures were incubated with serial fourfold dilutions of interferon in the usual manner and challenged with NDV_o, NDV_{pi}, or VSV at an input MOI of 1 as described in the text. The culture fluid was harvested after 24 hr and assayed for infectivity in CE cell monolayers. Cultures not treated with interferon were used as control. A plaque reduction assay was also carried out simultaneously as described in the legend of Fig. 1. The yield (PFU/ml) of progeny virus harvested from control cultures was 2.4×10^8 for VSV, 3.4×10^7 for NDV_o, and 1.2×10^7 for NDV_{pi}. Symbols: plaque reduction (broken line), yield reduction (solid line); for NDV_o (□ or ■), NDV_{pi} (△ or ▲), and VSV (○ or ●).*

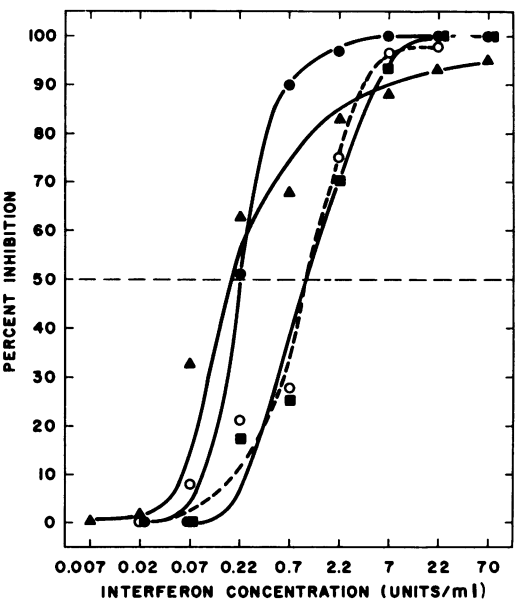

FIG. 3. *Inhibition of NDV and VSV by interferon in CE cell cultures: single-cycle yield reduction method. Cell cultures were exposed to interferon and challenged with NDV_o (■), NDV_{pi} (▲), or VSV (●) at an input MOI of 1 as described in the text. The culture fluid was harvested at 6 hr for VSV and 10 hr for NDV_o and NDV_{pi}. Cultures not treated with interferon were used as control. A plaque reduction test using VSV (○) was carried out simultaneously for the determination of interferon concentrations used in the experiment. The yield (PFU/ml) from control cultures was 8.0×10^6 for NDV_o, 3.7×10^5 for NDV_{pi}, and 6.1×10^7 for VSV.*

in plaque numbers but no inhibition of multiple-cycle yield. With NDV_{pi}, 500 units of interferon reduced plaque formation and multiple-cycle yield by 72% and 20%, respectively; the same amount of interferon produced complete inhibition of plaque formation and multiple-cycle yield by VSV.

The relative sensitivities of the three viruses to inhibition by interferon was also tested by the single-cycle yield reduction method. CE cell cultures were exposed to interferon and then challenged with the three viruses, as described above. The culture fluid was harvested after the time required for one cycle of replication, i.e., 6 hr for VSV and 10 hr for NDV_o and NDV_{pi}. The per cent inhibition of yield of each virus by interferon is shown in Fig. 3. These experiments point out that, when tested by inhibition of a single cycle of replication, all the viruses were at least as sensitive to interferon as the comparison standard, VSV plaque reduction. This finding is in striking contrast to the wide variations noted in the sensitivity of the different viruses when tested by the plaque reduction method in CE cell cultures (Fig. 1). It is difficult, in the light of these results, to designate NDV_o a "resistant" virus since 1 unit of interferon, determined by the VSV plaque reduction data, reduced single-cycle yield by 50%.

Inhibition of NDV_{pi} and VSV by interferon in L cell cultures. In view of the variations in sensitivity of NDV and VSV to interferon in CE cell cultures, experiments were carried out to study the inhibition of these agents by interferon in L cells. By using the plaque reduction method, the sensitivity of NDV_{pi} and VSV to interferon was determined in the usual manner. NDV_o was not included in this experiment since it does not produce plaques in L cells. Figure 4 shows that, by the plaque reduction method in L cells, NDV_{pi} was slightly more sensitive than VSV to inhibition by interferon. This is in sharp contrast to the results in CE cell cultures (Fig. 1); in CE cells,

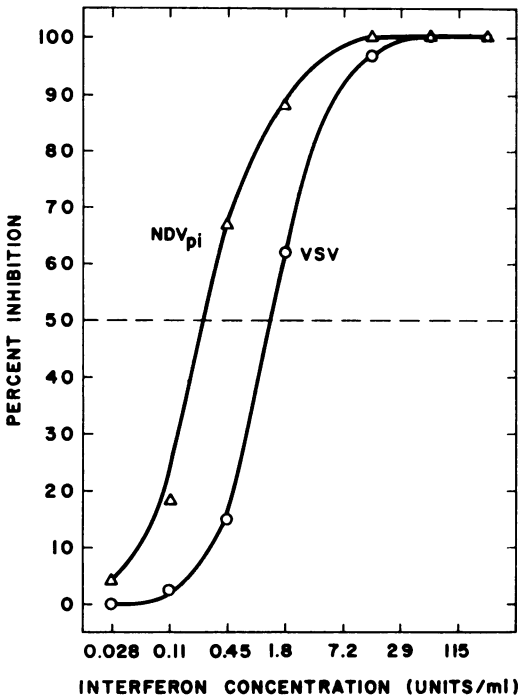

FIG. 4. *Inhibition of NDV_{pi} and VSV by interferon in L cell cultures. Cell cultures were incubated with various concentrations of interferon and challenged with 40 to 60 PFU of NDV_{pi} (△) or VSV (○) as described in the legend of Fig. 1.*

NDV_{pi} was found to be 15 times more resistant to interferon than VSV. These results indicate that the relative resistance of a virus to interferon, as measured by the plaque reduction method, is determined in large part by the host cell in which the tests are performed. Stewart and his colleagues (13), who studied the relative sensitivities of five different viruses in five different host cell systems, came to the same conclusion.

Single-cycle yield reduction tests were also carried out in L cells with NDV_{pi} and VSV as the challenge viruses. The results of these tests showed that NDV_{pi} was slightly more sensitive than VSV to inhibition by interferon, a result not unexpected considering the data obtained by the plaque reduction method. An explanation was sought for the marked effect of the host cell on the sensitivity of NDV and VSV to inhibition by interferon when measured by the plaque reduction method, a technique which involves multiple cycles of viral replication over a period of 3 days. Based on reports by several investigators (1, 2, 7, 16), it seemed possible that the resistance conferred upon CE cells by chick interferon was gradually lost during this time, allowing virus replication and plaque formation, whereas the resistance conferred upon L cells by mouse interferon was stable over the period of days required for plaque formation. To test this possibility, the following experiment was carried out.

Resistance of CE and L cell cultures to challenge with VSV at different times after removal of homologous interferon: plaque reduction method. Serial twofold dilutions of chick and L cell interferons were prepared, and each dilution was added to a large number of homologous cell cultures. Control cultures received medium only. After overnight incubation at 37 C, the interferon dilutions and the medium were removed, replaced with 3 ml of medium, and incubation was continued at 37 C. At the time that the interferon dilutions were removed from the cultures and at 4, 8, 12, 16, and 24 hr after their replacement with medium, a dilution series of each cell type was challenged with 40 to 60 PFU of VSV in the usual manner. By this procedure, the plaque reduction titer of the interferon was determined at different times after removal of the inhibitor from the cells, and the rate of loss of cellular resistance could be measured. The data in Table 1 show that cellular resistance was lost more rapidly in CE cell cultures than in L cells. The experiments were terminated 24 hr after the interferon dilutions were removed since preliminary experiments showed that, when tested by the plaque reduction method, CE cell cultures completely lost resistance to VSV by 48 hr after removal of interferon, whereas L cells retained most of their resistance at this time.

Resistance of CE cell cultures to challenge with NDV_o or VSV at different times after removal of interferon: single-cycle yield reduction method. CE cell cultures were incubated overnight at 37 C with 1,000 units of chick interferon or with medium alone. The fluids were removed, the cells were washed twice with medium, 3 ml of fresh medium was added, and the cultures were again incubated at 37 C. On the day the interferon was removed, and on each successive day, cultures were challenged with VSV or NDV_o at an input MOI of 10. Fluids from infected cultures were harvested after one cycle of replication and assayed for infectivity in CE cell monolayers. A striking difference was found in the rate of loss of resistance when measured by using different challenge viruses (Fig. 5). In the case of VSV challenge, CE cells maintained their resistant state almost unchanged for the 3-day period of the experiment. On the other hand, there was a rapid loss of resistance to NDV_o. Twenty-four hours after removal of the 1,000 units of interferon, single cycle yields of NDV_o were inhibited 71% instead of the 96% inhibition measured immediately after interferon was removed from the cultures. Two days after removal of interferon,

TABLE 1. *Resistance of chick embryo (CE) and L cells to challenge with vesicular stomatitis virus at different times after removal of homologous interferon*[a]

Cultures	Plaque-inhibition titer of interferon at					
	0 hr	4 hr	8 hr	12 hr	16 hr	24 hr
CE cells	1,400 (100)[b]	1,100 (78)	1,400 (100)	900 (64)	520 (37)	110 (8)
L cells	12,000 (100)	10,000 (83)	11,000 (92)	11,000 (92)	7,200 (60)	8,500 (71)

[a] CE or L cell cultures were incubated overnight at 37 C with twofold dilutions of the appropriate interferon preparation. The interferon dilutions were removed and replaced with 3 ml of medium, and incubation was continued. Groups of cultures were challenged with 50 PFU of VSV at different times after the interferon dilutions were removed.

[b] Numbers in parentheses represent the per cent of titer at 0 hr.

only a 13% inhibition of yield was recorded; at 3 days the interferon-treated cultures were as susceptible to NDV_0 as the untreated controls.

Effect of concentration of interferon on single-cycle yields of VSV or NDV_0 in CE cell cultures. The experiments which have been described show that the apparent "sensitivity" of a given virus to interferon depends on such factors as the virus mutant, the host cell employed, and the type of assay performed. The following experiment was carried out in an attempt to minimize the influence of loss of resistance in defining the sensitivity of NDV_0 and VSV to interferon and to establish a realistic basis for expressing the relative sensitivities of these viruses.

CE cell cultures were exposed to various concentrations of interferon and challenged with NDV_0 or VSV at an input MOI of 1, in the usual manner. Culture fluids were harvested after one cycle of replication and assayed for infectivity in CE cell monolayers. The inhibition of single cycle yields of NDV_0 and VSV is plotted in Fig. 6. The maximum level of inhibition of yield of both VSV and NDV_0 was produced with about 200 units of interferon per culture; higher concentrations of interferon did not further decrease the yields. However, there was a significant difference in the breakpoint of the inhibition curves of the two viruses. In the case of VSV, the maximum inhibition of yield was 4.0 logs, whereas with NDV_0 the maximum inhibition was 1.7 logs. This characteristic difference in maximum inhibition of NDV_0 and VSV was experimentally reproducible and probably is an accurate measure of their relative sensitivities to interferon. Experiments were performed which ruled out the possibility (i) that the NDV_0 population contained interferon-resistant variants and (ii) that a large population of reversibly adsorbed NDV_0 particles was eluted.

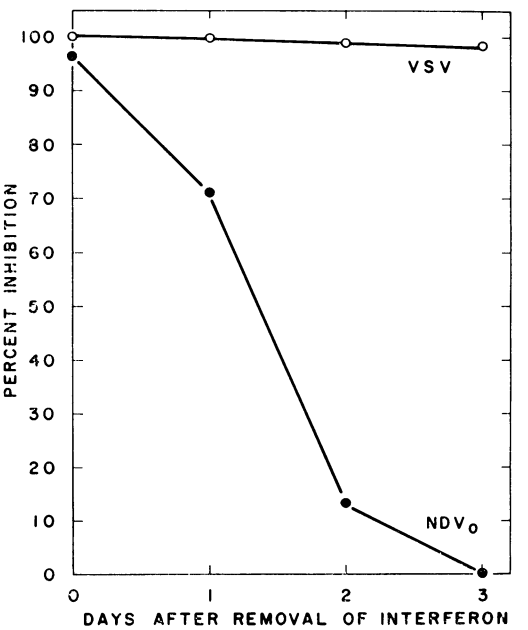

FIG. 5. *Resistance of CE cell cultures to challenge with NDV_0 or VSV at different times after removal of interferon: single-cycle yield reduction method. CE cell cultures were incubated overnight at 37 C with 1,000 units of interferon or with medium alone. Cultures were washed, refed with 3 ml of medium, and reincubated at 37 C. At various times after removal of interferon, the cultures were challenged in the usual manner with either VSV (○) or NDV_0 (●) at an input MOI of 10. Culture fluid was harvested after one cycle of replication and assayed for infectivity in CE cell monolayers. The yield (PFU/ml) of progeny virus from control (untreated) cultures was as follows: day 0, VSV = 10^5, NDV_0 = 1.05×10^7; day 1, VSV = 10^6, NDV_0 = 2.1×10^7; day 2, VSV = 1.6×10^7, NDV_0 = 2.4×10^7; day 3, VSV = 5.0×10^6, NDV_0 = 1.8×10^7.*

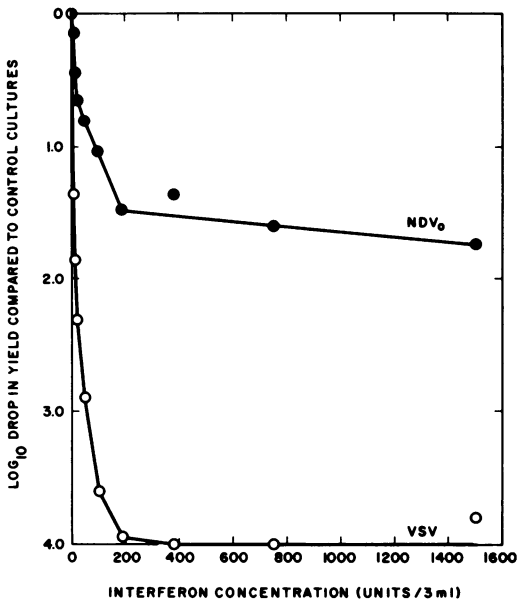

FIG. 6. *Effect of concentration of interferon on single-cycle yields of NDV_0 or VSV in CE cell cultures. Cell cultures were incubated overnight at 37 C with various concentrations of interferon and challenged in the usual manner with either VSV (○) or NDV_0 (●) at an input MOI of 1. Cell cultures without interferon were used as controls. Culture fluid was harvested after one cycle of replication and assayed for infectivity in CE cell monolayers. The yield (PFU/ml) of progeny virus from control cultures was 7.4 × 10^6 for NDV_0 and 3.0 × 10^7 for VSV.*

In the latter experiment, anti-NDV serum (18) was employed to neutralize all reversibly adsorbed virus prior to virus replication.

DISCUSSION

The experiments we have described show that the sensitivity of different viruses to inhibition by interferon in a given host cell system depends on the method of assay employed. For example, large differences in the sensitivities of NDV and VSV to interferon in CE cells were observed when methods were used which permitted multiple cycles of virus replication to occur. When test methods were employed which involved only single cycles of virus replication, these differences in sensitivity to interferon were obliterated. The major difference between single- and multiple-cycle replication methods is the elapsed time between infection and the harvest of progeny viruses. It is apparent from our data that the antiviral resistance established in CE cell cultures incubated with interferon decreased when the interferon was removed from the cells. The rate of decrease of antiviral resistance was different for NDV and for VSV. The more rapid disappearance of cellular anti-NDV activity with time furnishes an explanation for the observation that this virus is apparently "resistant" to inhibition by interferon when tested by the plaque or multiple-cycle reduction methods. On the other hand, when tested by the single-cycle yield reduction method, which minimizes the decay of established antiviral activity, NDV appears to be as "sensitive" to inhibition by interferon as VSV, a virus considered highly sensitive to interferon.

Our experiments also identify one of the factors which determine variations in sensitivity of a given virus when tested in different host cells by the same method of assay. When tested by the plaque reduction method in CE and L cell cultures, NDV_{pi} and VSV differ greatly in their relative sensitivity to interferon. In CE cells, 15 times more interferon is required to inhibit NDV_{pi} than to inhibit VSV. In contrast, in L cell cultures there is no significant difference in the inhibition of NDV_{pi} and VSV by interferon when tested by the plaque reduction method. Again, the factor which determines the "sensitivity" of a given virus to interferon is the rate of decay of cellular resistance when interferon is removed; decay of resistance is much more rapid in CE cells than in L cells.

The variations in rate of decay of cellular antiviral resistance when interferon is removed may explain the observations of Stewart et al. (13). These workers found significant differences in the sensitivity of five viruses to inhibition by interferon in cells from five different species. Since they employed the plaque reduction method, conditions were provided which allowed rate of decay of cellular resistance to play a major role in their experiments. Based on our experiences with CE and L cells, it seems reasonable to suggest that wide variations in the rate of decay of resistance would be encountered in the five species of cells which they employed.

The finding that, in CE cell cultures treated with interferon, resistance to NDV and VSV waned at different rates after the removal of interferon was unexpected. Two alternate hypotheses can be advanced to explain the finding that CE cells treated with interferon can remain resistant to VSV at a time when they have become completely susceptible to NDV_0. The first hypothesis is quantitative and suggests that a greater number of a single species of intracellular resistance factor induced by interferon is needed to inhibit NDV than to inhibit VSV. Initially, after incubation with interferon, there is a sufficient number of these factors to inhibit both viruses. As decay proceeds, the number of resistance factors per

cell would decrease to a level which, though still great enough to inhibit VSV, no longer is sufficient to block replication of NDV. The second hypothesis is qualitative and suggests that more than one species of intracellular resistance factor is induced by interferon. These different species vary in their ability to inhibit different viruses and decay at different rates. Thus, if two resistance factors were induced in CE cells by interferon, one factor active against VSV and the other aganst NDV (the latter less stable than the former), the results described in Fig. 5 would be obtained. Cell cultures would remain resistant to VSV at a time when they had become completely susceptible to NDV. In support of the qualitative hypothesis, one can cite reports that interferon preparations often contain two or more molecular species of inhibitor (5, 6, 8, 12). It seems possible that each of these molecular species could induce a different intracellular resistance factor. At present, our data do not permit a choice between the quantitative and qualitative hypotheses.

The closest we can come to the definition or expression of the differences in sensitivity of viruses to interferon is the following. By using the single cycle yield reduction method (which minimizes the role of decay of intracellular resistance factors) and by testing concentrations of interferon which produce maximum inhibition of yield, a "sensitivity factor" can be calculated. This factor for a given virus (NDV_o) is defined as the ratio of the $\log_{10}$ drop in yield compared to the $\log_{10}$ drop in yield of a standard, interferon-sensitive virus such as VSV. In the particular case shown in Fig. 6, this "sensitivity factor" for NDV_o (2.35) was calculated as follows: (maximum $\log_{10}$ drop for NDV_o/maximum $\log_{10}$ drop for VSV) = (4.0/1.7) = 2.35. Considering the number of variables which influence the inhibition of viruses by interferon, the method suggested for calculating the "sensitivity factor" may permit a more rational ranking of viruses in regard to their sensitivity to interferon than has been available previously.

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