INTERFERON INDUCTION WITH NEWCASTLE DISEASE VIRUS IN FS-4 CELLS: EFFECT OF PRIMING WITH INTERFERON AND OF VIRUS INACTIVATING TREATMENTS

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SUMMARY: Inoculation of human FS-4 cells with Newcastle disease virus (NDV) resulted in the induction of two distinct interferon responses, one that peaked at about 5 hr (early response) and one that reached a maximum between 10 to 24 hr after inoculation (second response). The early interferon response was enhanced by previous treatment of the cells with interferon (priming), whereas the second response decreased after interferon treatment in a dose-dependent manner. The early response diminished with decreasing multiplicities of infection, the magnitude of the second response in unprimed cells was relatively independent of the dose of NDV employed. The early interferon response was sensitive to inhibition by actinomycin D for only 1 hr after inoculation. In marked contrast, the second response remained sensitive to inhibition by actinomycin D until 12 hr after inoculation. The ability of NDV to induce the second response was greatly diminished by irradiation of the virus with ultraviolet light or by its treatment with hydroxylamine, whereas the ability to stimulate the early response was relatively resistant to these virus-inactivating treatments. Treatment of NDV with hydroxylamine abolished the virus to induce the second response at the same rate as it destroyed infectivity. The results suggest the existence of at least two distinct mechanisms of interferon induction by NDV; the early response is triggered either by a virion component or by a product of primary transcription, whereas induction of the second response requires the expression of some functions of the virus not needed for triggering the early response.

INTRODUCTION

The kinetics of interferon production after induction with Newcastle disease virus (NDV) or Polyinosinic-Polycytidylic acid [Poly (I). Poly (C)] in cultures of various rabbit or human cells are rather different. On exposure to Poly (I). Poly (C), interferon is induced early, with a peak in its rate of synthesis reached at about 3 to 4 hr after stimulation. Thereafter, production declines rapidly to an undetectable level (Kohase and Vilček, 1974; Vilček, 1970). In contrast, the peak of interferon production after induction with NDV usually is reached much later (Bausek and Merigan, 1970; Mozes and Vilček, 1975; Stewart, Gresser and Lockart, 1971b).

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It was reported in an earlier study from this laboratory (Mozes and Vilček, 1975) that kinetics of interferon production in the human FS-4 cell strain after inoculation with NDV can be altered by the treatment of cells with interferon (priming) before virus inoculation. Cells primed by incubation with a high dose of interferon for 6 hr before virus inoculation produced a distinct peak of interferon at about 5 hr after inoculation. Another, smaller peak of interferon production was discernible in primed cells at about 48 hr after virus inoculation. In contrast, a single broad peak of interferon production was seen in unprimed FS-4 cells with a maximum reached at about 15 hr.

In this paper interferon production in the first 10 hr after NDV inoculation is referred to as *early response*. All interferon production between 10 to 72 hr is termed *second response*; the latter term is used in the interest of consistency, since in the earlier publication (Mozes and Vilček, 1975), we used the term "later response" specifically to designate interferon production occurring in interferon-treated cultures between 24 and 72 hr. The "second response" thus includes the bulk of interferon produced after NDV inoculation in unprimed cells as well as late interferon production in primed cells that is sometimes discernible as a separate peak at about 48 hr.

In this study we analyzed the optimal conditions for the induction of the early and second responses in FS-4 cells inoculated with NDV and we found that they were markedly different. We conclude that triggering of the two responses is due to different virus functions.

MATERIALS AND METHODS

Cell cultures: FS-4 strain of human diploid fibroblasts, derived in this laboratory from a single neonatal foreskin, was used for interferon production in all experiments (Havell and Vilček, 1972). The cells were grown in Eagle's minimum essential medium (MEM), buffered with N-2-hydroxyethylpiperazine-N'2'-ethansulfonic acid (HEPES, 10 mM) containing 100 μ g of gentamicin and 2.5 μ g of fungizone per ml, and supplemented with 5% heated fetal bovine serum (FBS). All experiments were done in duplicate 12-day-old cultures grown to confluency in 60-mm plastic petri dishes in a humidified 36 C incubator provided with 5% CO₂.

Chick embryo cells prepared from 11-day-old embryos were employed in the plaque assay of NDV.

Preparation and purification of virus: NDV (Hickman strain) was propagated by intraallantoic inoculation of 11-day-old chick embryos. Clarified infected allantoic fluid was centrifuged for 2 hr at 90,000 $\times$ g in a Spinco 65 angle head rotor. The supernatant was discarded and the pellet was resuspended by gentle sonication in a small volume of TNE buffer (50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 7.3). The suspension was layered on 15–60% (w/v) linear sodium potassium tartarate gradient buffered to pH 7.3 with TNE, and then centrifuged to equilibrium in a Spinco SW 41 rotor (14 to 18 hr at 73,000 $\times$ g).

The virus band was diluted about 10 times and recentrifuged through 40% sucrose (7 ml) in TNE buffer at $80,000 \times g$ for 3 hr at 20 C. The pellet was resuspended in a small volume of TN buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.3) and the virus suspension, dialyzed in TN buffer for 3 days at 4 C, was stored at -80 C. Highly purified virus prepared by this method contained approximately 5×10^{10} plaque forming unit (PFU) per mg protein. Protein content was determined by the method of Lowry et al. (1951). Such highly purified virus was used in the experiments shown in Figs. 6 and 7. For other experiments NDV was partially purified by the method of Laver (1969) by adsorption of the virus to chick red cells at 0 C followed by elution into the medium.

Interferon production: FS-4 cell cultures were exposed to the appropriate dilutions of NDV or to 100 $\mu\text{g/ml}$ of Poly (I). Poly (C) in serum-free MEM containing HEPES (10 mM), 100 μg of gentamicin and 2.5 μg of fungizone per ml. Incubation of cells with the inducer was for 1 hr unless otherwise indicated. The cultures were then washed 3 to 5 times with Eagle's balanced salt solution (EBSS) to remove unadsorbed virus or Poly (I). Poly (C) and replenished with 2 ml of production medium consisting of MEM supplemented with 2% FBS. Culture fluids were collected after the appropriate times of incubation at 36 C. Virus-induced samples were always adjusted to pH 2 and kept at 4 C for 4 to 5 days to inactivate infectious NDV. Interferon used for the treatment of cultures was produced in FS-4 cells by stimulation with Poly (I). Poly (C) and superinduction with cycloheximide and actinomycin D as described (Vilček, 1970; Vilček, Rossman and Varacalli, 1969).

Interferon assay: The method for titration of human interferon in 96-well plastic trays was described in detail (Havell and Vilček, 1972). For all interferon assays we employed a strain of human diploid fibroblast (FS-7) derived in this laboratory and vesicular stomatitis virus. All interferon titers are expressed as reference units, calibrated against the human leukocyte interferon reference standard G-023-901-527 obtained from the Antiviral Substances Program, National Institute of Allergy and Infectious Disease, Bethesda, MD.

Ultraviolet (UV) irradiation of NDV: A General Electric G15T8 germicidal fluorescent lamp was used for the irradiations. NDV was suspended in 3 ml of phosphate-buffered saline (PBS) placed in open 60-mm petri plates and irradiated from the distance of 40 cm. Calibration by an Eppley thermopile indicated a dose rate of 34.9 $\text{erg/mm}^2/\text{sec}$ at the target distance of 40 cm.

Inactivation of virus by hydroxylamine (HA): The method used was essentially as described by Skehel and Burke (1968). A solution of HA (0.4 M) in PBS, adjusted to pH 7.2 with 2 M NaOH, was freshly prepared before use. Highly purified virus was diluted to 2×10^8 PFU/ml in PBS and an aliquot of 0.3 ml was mixed with an equal volume of the HA solution (final concentration 0.2 M). The reaction was allowed to proceed at 37 C for the indicated times and was stopped by chilling the mixture in ice-cold water by adding 2.4 ml of MEM. To remove residual HA, all samples were then dialyzed at 4 C for 24 hr with two changes.

Fig. 1. The kinetics of interferon production in primed and unprimed FS-4 cells inoculated with NDV. Cultures were incubated for 6 hr with interferon (400 unit/ml) (●) or with control medium (○). All cultures were inoculated with NDV (exposed to UV irradiation, 700 erg/mm², and diluted to give input multiplicity of 100 plaque-forming units per cell as calculated from the titer of unirradiated virus). Adsorption of NDV was for 2 hr. Culture fluids were collected at the indicated intervals. After each collection, the cells were washed once with EBSS, replenished with fresh production medium warmed to 36 C and reincubated until the next sampling. Interferon titers are expressed in reference units/ml divided by the number of hours that had elapsed from the preceding interval. All incubations were at 36 C.

Chemicals: Poly (I). Poly (C) was supplied by the Antiviral Substances Program, Infectious Disease Branch, National Institute of Allergy and Infectious Disease, Bethesda, MD. Actinomycin D was purchased from Calbiochem, Los Angeles, CA, and HA-HCl from Fisher Chemical Co., Springfield, NJ.

RESULTS

Kinetics of Interferon Production in Primed and Unprimed Cells

FS-4 cell cultures were either primed by incubation with interferon (400 units/ml for 6 hr) or incubated with control medium, and then inoculated with NDV (Fig. 1). As already reported (Mozes and Vilček, 1975), interferon production in unprimed cells rose gradually and reached a maximum between 10 to 24 hr after inoculation. In contrast, interferon production in primed cells peaked at 5 hr. Thus the early response was enhanced but the second response diminished in primed cells.

In a related experiment we chose 16-hr incubation with interferon priming, using various interferon concentrations ranging from 1 to 10,000 units/ml

Fig. 2. Effect of priming with various doses of interferon on early and second responses. Cultures were incubated with various doses of interferon for 16 hr, and the cells were then inoculated with UV-irradiated NDV as described in Fig. 1. To measure the early response interferon yields were determined in culture fluids collected at 7.5 hr. Fresh production medium was added to all cultures at 7.5 hr and again at 10 hr. The second response represents interferon accumulated in the culture medium between 10 and 48 hr after inoculation.

(Fig. 2). As little as 1 unit/ml or as much as 1,000 units/ml produced marked priming of the early response. Interferon concentrations over 1,000 units/ml caused less priming or no priming at all. The second response was not significantly affected by 1 or 3 units/ml but incubation of cells with higher interferon doses produced a gradual decrease in the amount of interferon produced during the latter time period.

Since priming with very low doses of interferon increased the early response without decreasing the second response, we examined the kinetics of interferon production in cultures primed with 3 units/ml (Fig. 3). The only significant difference between the primed and unprimed groups in this experiment was a marked increase in the early response.

Differential Effect of Actinomycin D on the Early and Second Interferon Responses

The results of the preceding experiments suggested that the induction of the two responses after NDV inoculation is due to distinct processes that can be separated from each other. Since interferon production requires cellular mRNA synthesis, treatment with a high dose of actinomycin D before induction or during the early stages of induction invariably inhibits interferon synthesis (Heller, 1963; Wagner, 1963). We decided to determine the duration of the actinomycin D-

Fig. 3. The kinetics of interferon production in cells primed with a low dose of interferon. Cultures were incubated with 3 units/ml of interferon (●) or with control medium (○) for 16 hr and then inoculated with NDV. The details of inoculation and determination of the kinetics of interferon production were as described in Fig. 1.

sensitive period during the induction of the early and second responses.

The early interferon response was measured in cultures primed with interferon and the second response was analyzed in unprimed cells. For comparison we employed also interferon induction with Poly (I). Poly (C) in which actinomycin D resistance is known to develop within 1 to 2 hr after induction (Vilček, 1970; Vilček and Havell, 1973). A comparison of the kinetics of interferon accumulation in the absence of actinomycin D (Fig. 4A) showed that the rise in interferon was the fastest in cultures stimulated with Poly (I). Poly (C), reaching a peak by 4 hr—as also shown in previous studies (Havell and Vilček, 1972; Vilček and Havell, 1973). Treatment with actinomycin D before induction inhibited all three interferon responses (Fig. 4B). However, both Poly (I). Poly (C)-induced interferon production and the NDV-early response were no longer inhibited by actinomycin D treatment at 2 hr, whereas the second response did not become completely resistant to actinomycin D treatment until 12 hr after inoculation. Although Poly (I). Poly (C)-induced interferon production and the early response showed some similarities, the former was increased about fivefold by treatment with actinomycin D at 3 or 4 hr, whereas no such marked enhancement was seen in the early response. A similar increase in Poly (I). Poly (C)-induced interferon production after actinomycin D treatment was reported earlier (Vilček, 1970; Vilček *et al.*, 1969).

The results of this experiment suggest that interferon mRNA synthesis for the second response is not completed until about 12 hr after NDV inoculation.

Fig. 4. Kinetics of interferon production and the effect of actinomycin D on early response, second response and Poly (I). Poly (C)-induced response. Panel A shows the kinetics of interferon accumulation in cultures induced with Poly (I). Poly (C), in cultures primed by incubation with interferon (100 unit/ml) for 16 hr and then incubated with NDV as described in Fig. 1 (early response), and in unprimed cultures inoculated with NDV as described in Fig. 1 (second response). Incubation of cells with NDV and Poly (I). Poly (C) was for 1 hr in all groups. To measure interferon accumulation, small aliquots of medium were collected at the indicated times. Panel B shows the effect of actinomycin D on the three interferon responses examined in parallel. In addition to induction by the appropriate method, cultures were treated with actinomycin D (final concentration $2 \mu\text{g/ml}$) as follows. At -1 hr, treatment in all groups was for 1 hr. To determine the effect of actinomycin D on the early response and the Poly (I). Poly (C)-induced interferon production, actinomycin D was added to the culture fluids at the indicated times after induction; culture fluids were collected at 10 hr and interferon yields were determined after removing actinomycin D by thorough dialysis. To examine the effect on the second response, actinomycin D was added for 1 hr at the indicated times, the cells were washed, replenished with medium and further incubated. Interferon accumulated in the culture medium between 13 and 24 hr after NDV inoculation was measured.

Effect of Different Multiplicities of Infection on Relative Magnitude of Early and Second Interferon Responses

Cultures were either primed by 16-hr treatment with interferon or incubated with control medium. Thereafter both groups of cultures were inoculated with different dilutions of live, unirradiated NDV, resulting in input multiplicities of infection ranging from 0.1 to 100 PFU per cell (Fig. 5). The early interferon response was diminished with decreasing multiplicities of virus infection in both primed and unprimed cells. The second interferon response in interferon-treated cells was also diminished with decreasing multiplicities of infection. In contrast, the magnitude of the second response in unprimed cells was relatively independent of the dose of NDV used for inoculation.

Fig. 5. Effect of multiplicity of infection with NDV on the early and second interferon responses. Cultures were incubated with interferon (100 units/ml) or with control medium for 16 hr and then inoculated with various doses of unirradiated NDV. Adsorption of virus was for 2 hr. Culture fluids were collected at 6 hr (early response). The second response represents interferon accumulated in the culture fluids between 10 and 48 hr.

Fig. 6. Effect of UV irradiation on the ability of NDV to stimulate the two interferon responses. Highly purified NDV prepared as described in Materials and Methods was irradiated with various doses of UV and immediately thereafter used for the inoculation (30 plaque forming units/cell as calculated from the titer of unirradiated virus) of primed (100 interferon units/ml, 16 hr) or unprimed cultures. The early and the second interferon responses were determined as described in Fig. 5.

Fig. 7. Effect of inactivation with hydroxylamine on the ability of NDV to stimulate the two interferon responses. Highly purified NDV was treated with hydroxylamine as described in the Materials and Methods and tested for interferon induction in primed cells (early response) or in unprimed cultures (second response) as in the experiment shown in Fig. 6.

*Effect of Virus-Inactivating Treatments on the Ability of
NDV to Induce the Two Distinct Interferon Responses*

The experiments shown in Fig. 6 were done to determine the effect of a wide range of UV doses on the ability of NDV to induce the early and second responses. UV irradiation caused a marked decrease in the second response in cells not treated with interferon. The second response in primed cells was also decreased, particularly with higher UV doses. However, the early response was affected to a much smaller extent, if at all. At a certain range of UV doses the early response in unprimed cells appears to have been increased somewhat over that seen with unirradiated virus. Although this increase was no more than twofold, it was seen in another, independent experiment.

Hydroxylamine inactivates viruses through reaction with virion nucleic acid (Franclin and Wecker, 1959; Skehel and Burke, 1968). This drug reacts with cytosine or uracil with resulting inactivation of template function (Phillips et al., 1965). It was of interest to compare the effect of HA treatment on the ability of NDV to induce interferon with that of UV irradiation, since both treatments affect primarily the nucleic acid of the virus, though by different mechanisms. With HA the rate of inactivation of virus infectivity paralleled exactly the rate of inhibition of the second interferon response. It appears that both curves deviated slightly from first order kinetics at their tail ends (Fig. 7). Compared to UV irradiation, HA was more effective in inhibiting the second response. As with UV irradiation, the ability of virus to induce the early response was relatively resistant to inactivation.

DISCUSSION

The mechanism of interferon induction by viruses is not fully understood. One of the missing pieces of information is the nature of the triggering event responsible for induction. Since isolated double-stranded RNA is a potent interferon inducer, some form of hydrogen-bonded RNA made during virus replication has been the major candidate for this function (Johnston and Burke, 1973). This view has been strengthened by the recent demonstration that a single defective interfering particle of vesicular stomatitis virus containing covalently linked complementary RNA can induce high levels of interferon in chick cells (Marcus and Sekellick, 1977). However, no clear correlation could be found between viral RNA synthesis and interferon induction in several studies that employed temperature-sensitive mutants of different viruses (Lai and Joklik, 1973; Lockart *et al.*, 1968; Lominiczi and Burke, 1970). Furthermore, as mentioned in the introduction to this paper, many differences were seen in the characteristics of interferon induction with isolated double-stranded RNA and with viruses (Bausek and Merigan, 1970; Mozes and Vilček, 1975; Stewart *et al.*, 1971b; Vilček and Havell, 1973).

An earlier study from this laboratory showed that NDV elicits two different interferon responses in FS-4 cells (Mozes and Vilček, 1975). Interferon production in this system peaked at about 15 hr after inoculation. UV irradiation of cells at the time of inoculation with NDV decreased interferon production. However, a small fraction of the total interferon produced at about 5 hr after inoculation, was resistant to UV irradiation of producing cells. This UV-resistant early response was enhanced by the treatment of cells with interferon (priming), unlike the major peak that was decreased after treatment of cells with interferon. Furthermore, treatment of FS-4 cells with an inhibitor of RNA synthesis, DRB exerted a differential effect on the two interferon responses (Kohase and Vilček, 1979). It was concluded that NDV may induce interferon by more than one mechanism.

This paper contains additional evidence for the existence of two distinct triggering mechanisms of interferon production by NDV. Not only was the early response enhanced and the second response inhibited by priming, but the magnitude of the two responses was also differently affected by varying the multiplicity of infection and by virus inactivating treatments.

It has been reported that treatment of cells with interferon before inoculation with viruses sometimes causes an increase and sometimes a decrease of subsequent interferon production depending on the experimental conditions employed (Barmak and Vilček, 1973; Stewart, Gosser and Lockart, 1971a). Since the enhancing effect is observed both with viral and nonviral inducers it probably represents in most instances a *direct* effect on the *cellular* capacity to respond to the inducer. The inhibitory action of interferon treatment on interferon production, on the other hand, may be only a secondary effect resulting from the inhibition of viral functions required for interferon induction.

The mechanism of the direct priming effect is not fully understood. Interferon is known either to increase or to decrease various cell functions. The enhancement of early interferon production by priming in the system studied by us may be an example of altered gene expression caused by interferon. On the other hand, inhibition of the second response in interferon-treated FS-4 cells most likely occurs as a result of inhibition of viral function(s) needed for triggering the second response.

What are the viral functions required for the induction of the two interferon responses? As judged from the dependence on the dose of NDV inoculated and resistance to virus-inactivating treatments, the early response is triggered either by a virion component or by a product of the virion transcriptase. The fact that the early response is relatively resistant to UV or HA treatment of the virus does not rule out the possibility that viral RNA synthesis is needed for induction of the early response since, at least in the case of UV, it is known that NDV transcriptase activity decays more slowly than infectivity and that the decay of transcriptase activity of NDV correlates with the decay of interferon-inducing activity in chick cells (Clavell and Bratt, 1971; Meager and Burke, 1972; Sheaff, Meager and Burke, 1972).

The second response, on the other hand, apparently requires the expression of some virion function(s). The nature of the function required for the induction of the second response remains unidentified. Any function after (or including) primary transcription of the virus genomes could be involved. However, infectious progeny is not likely to be required since FS-4 cells are not fully permissive for NDV, with less than 0.1 PFU being released per cell after inoculation with a high multiplicity of infection (Kohase and Vilček, 1979; Mozes and Vilček, 1975).

It is not obvious why HA inactivated the ability of virus to induce the second response at the same rate as virus infectivity, whereas UV destroyed much more rapidly the infectivity of the virus. Both agents are known to react primarily with nucleic acid. As already mentioned, primary transcription is not abolished in NDV by doses of UV that destroy infectivity (Clavell and Bratt, 1971). The effect of HA on the NDV transcriptase is not known. Should it turn out that transcriptase activity is strongly inhibited by HA (although there is no obvious reason for this), then our data could be explained by postulating that the early response is triggered by a virion component, whereas the second response requires primary transcription and possibly translation of some primary transcripts.

On the basis of all the results presented here and from the related papers, some characteristics of interferon responses in FS-4 cells induced by NDV or Poly (I). Poly (C) are summarized in Table I. Recent reports from other laboratories also suggested that more than one virus function is involved in the induction of interferon with reovirus in mouse cells (Lai and Joklik, 1973) and with NDV in chick embryo cells (Kowal and Younger, 1978).

Another example of heterogeneity of the virus-induced interferon response was recently reported from our laboratory (Havell and Vilček, 1978). Human fibroblast (F) cells were shown to produce not only the *F* interferon species that

TABLE I

Some characteristics of interferon responses induced by NDV or Poly(I). Poly(C) in FS-4 cells

Inducer	Peak of interferon synthesis (hr)	Time actinomycin sensitivity develops (hr)	Effect of priming with interferon	Effect of multiplicity of NDV infection	Effect of hydroxylamine or UV treatment of NDV	Effect of DRB	Effect of UV irradiation of cells
NDV (early response)	5	2	Enhancement	Increased with high multiplicity	Relatively insensitive	Enhancement ^a (superinduced)	No inhibition (possibly enhanced) ^b
NDV (second response)	10-24	12	Inhibition	Relatively independent of multiplicity of NDV	Decrease	Inhibition ^a	Inhibition ^b
Poly(I). Poly(C)	3	2	Enhancement ^b	NA ^c	NA	Enhancement (superinduced) ^d	Enhancement (superinduced) ^b

^a Kohase and Vilček, 1979

^b Mozes and Vilček, 1975

^c Not applicable

^d Sehgal, Tamm and Vilček, 1975.

is found after induction with Poly (I). Poly (C), but also small amounts of *Le* interferon. (*Le* interferon is the major interferon species produced in lymphoid cells). The proportion of *Le* interferon produced in FS-4 cultures after NDV inoculation was very small, in the order of 2% of total interferon activity produced. Preliminary data show that this small amount of *Le* interferon is produced during the second response (Hayes and Vilček, unpublished data).

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