Simultaneous Viral and Non-Viral Interferon Production in Human Cell Cultures[1] (34609)

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Recent studies by Vilček and associates (1, 2) suggest that an inhibitor of interferon formation is produced by cells to regulate the termination of interferon production by the double-stranded RNA polycytidylate: polyinosinate (poly C:I). Evidence for this concept came from a kinetic analysis of the effects of metabolic antagonists on the interferon response by poly C:I in rabbit kidney cells. It was found that late inhibition of RNA and protein synthesis resulted in a prolonged and elevated interferon response (1, 2). The present report describes experiments designed to investigate whether interferon induction by poly C:I would influence the simultaneous induction of interferon by virus. Previous studies from this laboratory (3) had pointed out differences in the induction of interferon by viral and nonviral agents and had suggested the feasibility of investigating the simultaneous induction of the two responses. On the basis of studies described in this paper, we were able to distinguish between viral and nonviral interferon in simultaneously stimulated cells. Our findings indicate that the viral interferon response was not affected by the concomitant induction of nonviral interferon.

Materials and Methods. The techniques used for the induction of interferon by poly C:I and Newcastle disease virus (NDV), the assay and characterization of interferon, and the culture of human foreskin fibroblasts have been described in earlier reports (3–5). Poly C and poly I were obtained either from Miles Laboratories, Elkhart, Indiana, or from P-L Biochemicals, Milwaukee, Wisconsin. They were annealed to form poly C:I as previously described (4). Fifty μg/ml of poly C:I and the Herts strain of NDV were used in the present studies. Before assay for interferon, samples containing viral interferon were incubated with rabbit anti-NDV serum in a ratio of 40:1 (v/v) for 20 min at 25° and centrifuged for 1 hr at 40,000 rpm using a Spinco T65 rotor. The incorporation of ^{3}H-uridine or ^{14}C-leucine into acid-insoluble material was measured by the following method. Confluent monolayers of human fibroblasts in 60-mm dishes were incubated for 30 min at 37° with either 2 ml of 1 μCi/ml ^{3}H-uridine (New England Nuclear Corp.) in Eagle's minimal essential medium (MEM) containing 1% bovine serum, or with 2 ml of 0.1 μCi/ml ^{14}C-leucine (Schwarz Bioresearch Inc.) in leucine-free MEM containing 1% bovine serum. The cells were then washed with cold phosphate-buffered saline and incubated for 15 min at 4° with 2 ml of 5% trichloroacetic acid. This was followed by briefly washing the cell monolayers with 0.9% saline and draining. 0.8 ml of Nonidet P40 (gift of Shell Oil Co., New York), 0.5% in distilled water, was then added, and the monolayers were scraped into scintillation vials. Radioactivity was measured in a liquid-scintillation counter using 10 ml of Bray's solution per vial.

For treatment with actinomycin D (gift of Merck, Sharp and Dohme), cell monolayers were incubated for 1 hr at 37° with 2 ml of 0.3 μg/ml actinomycin D in MEM. Puromycin (Nutritional Biochemicals Corp., Cleveland) was used at 5 μg/ml in MEM. It was noted that treatment of human fibroblasts with this dose of puromycin resulted in morphological cytotoxicity after 15–20 hr in-

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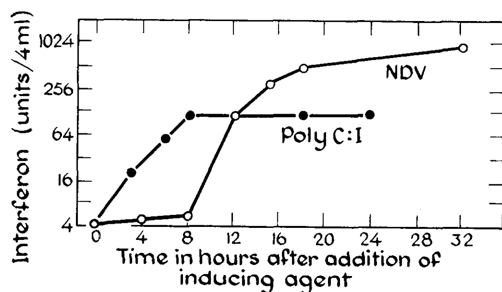

FIG. 1. Kinetics of interferon induction by poly C:I and NDV in human fibroblasts.

cubation. Unless specified otherwise, 12 hr were allowed for interferon induction by poly C:I and 18 hr for the interferon response by NDV. Control media consisted of MEM containing 1% bovine serum.

Results and Discussion. Characterization of viral and nonviral interferon production. Figure 1 shows the kinetics of the appearance of interferon in the cell supernatant fluid after stimulation with poly C:I (50 μg/ml) or infection with NDV. It is seen that 8 hr after the addition of poly C:I, the full yield of interferon was released, while only negligible amounts of interferon were observed at this time in NDV-infected cell cultures. On the other hand, only small amounts of additional nonviral interferon were detected after the 8-hr point, in contrast to the steep rise in viral interferon production (Fig. 1).

Actinomycin D and puromycin were used to additionally characterize the nonviral and viral interferon response in human fibroblasts. Figure 2a shows the effect of these agents, added at the indicated times, on interferon induction with poly C:I. The dose of actinomycin D used in our studies inhibited approximately 85% of ^{3}H-uridine incorporation in controls. This dose of actinomycin D (0.3 μg/ml) enhanced the interferon response by poly C:I at all points tested, particularly when administered at late times (Fig. 2a). Moreover, the interferon response by poly C:I was resistant to puromycin (5 μg/ml) in all cases, and enhanced when puromycin was given at late times. These findings are in agreement with those of Vilček and associates (1, 2) and support the concept that the late addition of metabolic antagonists may inhibit the synthesis of a repressor of interferon, thereby enhancing the nonviral interferon response.

Figure 2b summarizes our studies on the effects of actinomycin D and puromycin on viral interferon production. In contrast to poly C:I, interferon induction by NDV was found to be greatly inhibited by puromycin at all times. In further contrast to poly C:I, the viral interferon response was initially in-

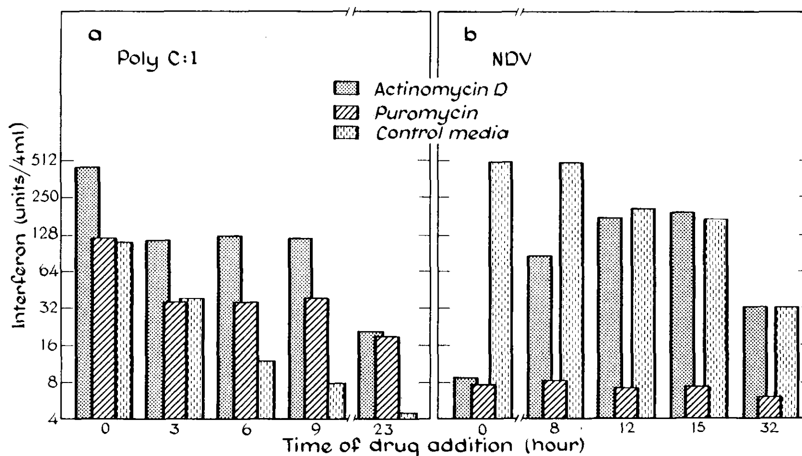

FIG. 2. Effect of actinomycin D and puromycin on interferon induction by poly C:I and NDV. Sets of cell monolayers were initially treated with the interferon inducer, then incubated at the indicated times for 1 hr at 37° with either actinomycin D (0.3 μg/ml), puromycin (5 μg/ml), or control media (MEM containing 1% serum). After this interval, the cell supernatant fluids were replaced with fresh control media, or 5 μg/ml puromycin in the case of puromycin-treated plates.

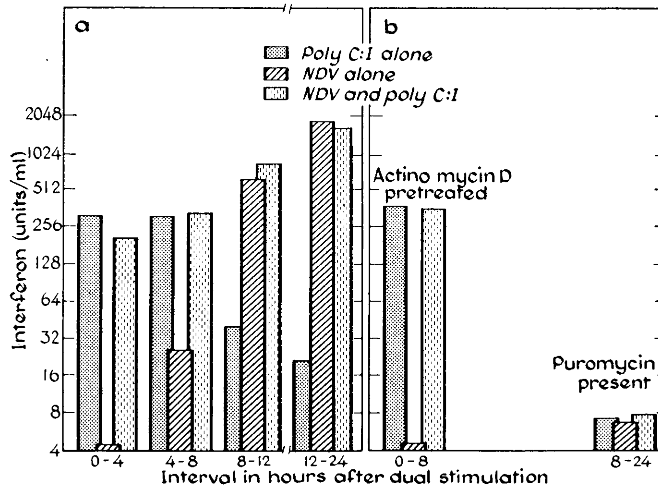

FIG. 3. Nonviral and viral interferon response in dually induced cell cultures. Cell monolayers were incubated for 1 hr at 37° with either control media or NDV as indicated. After this time, the cell supernatant fluids were replaced with either poly C:I (50μg/ml) or control media. The fluids were then collected in successive intervals as shown. Fresh media was added to the monolayers after each collection. In part b of the figure, the cell monolayers were either pretreated with actinomycin D before interferon induction, or treated with puromycin during the indicated interval.

hibited by actinomycin D and only became resistant when the drug was given more than 8 hr after viral infection. These data agree with earlier studies employing NDV by Wagner and Huang (6) and Ho and Breinig (7).

Simultaneous interferon induction by poly C:I and NDV. The results of Fig. 2a indicate that moderate inhibition of RNA and protein synthesis interfered with the normal termination of the poly C:I-induced interferon response. However, it could not be determined from such data whether the inhibitor acts as an "inactivator" of the inducer, or by either inhibiting the formation or enhancing the inactivation of interferon. As no physical or biological difference has been found between interferon induced by virus and by poly C:I (8), it seemed of interest to investigate whether the hypothesized inhibition of nonviral interferon would act on a simultaneous induction of interferon by virus. For this purpose cell monolayers were infected with NDV and/or stimulated with poly C:I 1 hr later, as indicated in Fig. 3a. The cell supernatant fluids were collected in three successive 4-hr intervals and, finally, after a 12-hr interval. Fresh control media was added to the cell monolayers after each fluid collection. It is seen in Fig. 3a that the release of viral interferon was not significantly affected by the late phase of the poly C:I-induced response. Control experiments, summarized in Fig. 3b, showed that interferon production in dually stimulated cells was resistant to actinomycin D during the first 8 hr and sensitive to puromycin between 8 and 24 hr. This result suggests that the poly C:I-induced interferon response was turned off shortly before the appearance of viral interferon in the extracellular medium.

The release of full yields of viral interferon immediately after the repression of nonviral interferon (Fig. 3) suggests that the inhibitor did not inactivate interferon directly. Instead, it apparently interfered with the continued formation of nonviral interferon at a metabolic step that is not shared by the viral interferon-induction mechanism. In view of the apparent similarity between nonviral and viral interferon (8), it seems unlikely that the hypothesized inhibitor could distinguish between viral and nonviral interferon and selectively inactivate poly C:I-induced interferon.

It has recently been reported (9) that certain strains of NDV inhibit both host RNA

TABLE I. Inhibition of Total RNA and Protein Synthesis in Human Fibroblasts by NDV (Herts strain).

Time after NDV infection (hr)	^{3}H-Uridine incorporation (% of uninfected control)	^{14}C-Leucine incorporation (% of uninfected control)
0.5	90	92
4.5	106	105
8.5	96	86
12.5	73	64
24.5	4	9

and protein synthesis several hours after infection. As a control experiment, we consequently investigated the effect of NDV (Herts strain) on RNA and protein synthesis in human fibroblasts. The results are shown in Table I and suggest that host RNA and protein synthesis were not significantly blocked during the first 8 hr after infection. Therefore, it appears unlikely that the virus would have prevented the synthesis of the hypothesized inhibitor.

Summary. Homogeneous monolayers of human fibroblasts were infected with Newcastle disease virus (NDV) and 1 hr later treated with the synthetic RNA polycytidylate: polyinosinate (poly C:I). The induction of interferon by poly C:I and NDV could be distinguished on the basis of differences in (1) the kinetics of interferon production, and (2) the sensitivity to inhibitors of RNA and protein synthesis. The results indicated that both poly C:I and NDV were fully able to induce the production of interferon in these dually stimulated monolayers. The release of interferon induced by poly C:I appeared to be terminated shortly before the appearance of viral interferon in the extracellular medium. Our findings suggest that termination of the nonviral interferon response did not involve direct inactivation of interferon. It appears that a inhibitor substance blocked the continued formation of nonviral interferon, without significantly affecting the induction of viral interferon.

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