Two Mechanisms of Cell Resistance to Repeated Stimulation of Interferon[1] (34858)

GERALD H. BAUSEK[2] AND THOMAS C. MERIGAN

Division of Infectious Diseases, Department of Medicine, Stanford University School of Medicine, Stanford, California 94305

When mouse L cells are stimulated to produce interferon with Newcastle disease virus (NDV), they become resistant to a second stimulation of interferon production by NDV (1). The factor responsible for this resistance could not be separated from interferon by a number of physical techniques. The present report describes studies on the development of resistance to interferon production after cell treatment with the double-stranded synthetic RNA, polyinosinate: polycytidylate (I:C), which also initiates the production of interferon (2). We found that I:C causes in human fibroblasts a state of resistance to stimulation of interferon production by either virus or a second dose of I:C. We shall use the term "tolerance" to refer to the state of cell resistance to repeated stimulation of interferon. Depending on whether NDV or I:C was employed as the second stimulating agent, the terms NDV-tolerance and I:C-tolerance will be used, respectively. NDV-tolerance appears to be mediated by interferon produced during treatment with I:C. However, a different mechanism appears to be responsible for I:C-tolerance.

Materials and Methods. Tissue culture and interferon. The techniques used for the culture of human foreskin fibroblasts, the production of interferon by I:C and NDV, and the assay and characterization of interferon have been described previously (3–5). Interferon was assayed by a plaque-reduction assay using vesicular stomatitis virus (VSV) as the challenge virus. Tissue culture medium (TCM) consisted of Eagle's minimal essential medium (MEM) supplemented with 3% calf serum.

Nucleic acids. The synthetic, single-stranded polynucleotides, polyinosinate (I) and polycytidylate (C), were obtained from P-L Biochemicals, Milwaukee, Wisconsin. I:C was prepared as described earlier (3) and used at a dose of 50 μg/ml for the production of interferon. H^3-C was supplied by Schwarz Bioresearch, Orangeburg, N. Y.

Virus. The Herts strain of NDV was used for interferon production at a multicplicity of 5 PFU/cell.

Actinomycin D treatment. Human fibroblasts were incubated for 1 hr at 37° with 2 ml of 0.3 μg/ml actinomycin D (gift of Merck, Sharp and Dohme, Rahway, New Jersey) in TCM. This dose of actinomycin D inhibited approximately 80% of ^{3}H-uridine incorporation in control cell monolayers and suppressed the stimulation of interferon by NDV but not by I:C, as reported earlier (6).

Interferon preparation for cell treatment. Human fibroblast interferon was prepared with NDV and lyophilized as described elsewhere (7).

Cell extracts. To assay for intracellular ribonuclease, cell extracts were prepared by the following method. Cell monolayers (2×10^6 cells per monolayer) were washed twice with phosphate-deficient saline before adding 2 ml of hypotonic buffer (0.01 *M* NaCl, 0.01 *M* Tris–HCl, pH 7.2, 0.0015 *M* $MgCl_2$; or 0.10 *M* Na acetate, pH 5.0). The cells were then scraped and Dounce homogenized with 10 strokes. EDTA was added to a final concentration of 0.001 *M*, and the extracts were incubated for 1 hr at 37° with 2 μg/ml I:C-^{3}H (0.01 μCi/μg). This was followed by analysis

[1] Supported by U.S. Public Health Service Grant A105629.

[2] Biophysics Program, Stanford University.

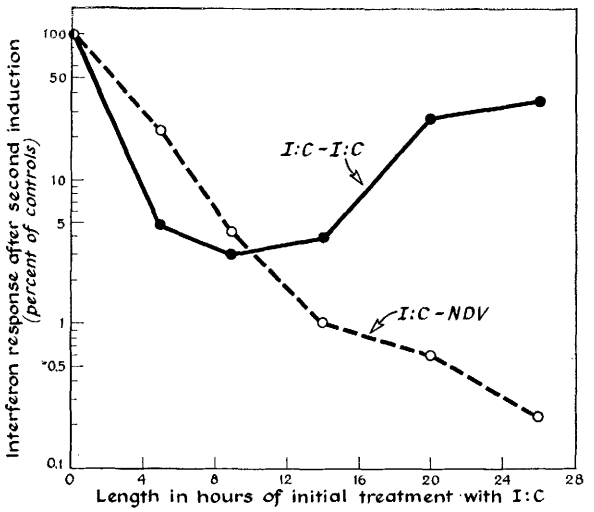

FIG. 1. Two mechanisms of hyporeactivity to repeated interferon stimulation. Human fibroblast monolayers were first treated with I:C for varying times, then washed and further incubated with either I:C (9 hr) or NDV (24 hr) before assay of the cell supernatant fluids for interferon. The interferon response after the second treatment is expressed as percentage of controls described in the text.

for acid-insoluble radioactivity as described earlier (3).

Results. Tolerance after cell treatment with I:C. To study the development of tolerance after treatment with I:C, human fibroblasts were incubated with 50 μg/ml I:C for various times ranging from 5–26 hr. After washing once with tissue culture medium (TCM), the cells were then stimulated either for 9 hr with a second dose of I:C or for 24 hr with NDV. The cell supernatant fluids were collected after this time and assayed for interferon. Two sets of controls were used. One set received TCM in place of the second interferon-stimulating agent, in order to determine the amount of interferon residually produced from the initial treatment with I:C. A second set of controls received TCM in place of the initial treatment with I:C, in order to show the amount of interferon produced by the second agent in previously unstimulated cells. Interferon production occurring as a residuum from the first stimulation was subtracted from the amounts of interferon in the cell supernatant fluid after the second induction. Figure 1 summarizes the results, expressed as percentage of the response obtained in the previously unstimulated set of controls. Interferon titers of 380 and 2000 units/4 ml were obtained with I:C and NDV, respectively, in the previously unstimulated controls. Treatment with I:C caused cell resistance to subsequent stimulation of interferon by either NDV or I:C. However, it appears that two types of tolerance occurred. Prolonged treatment with I:C (20 hr or more), reduced the tolerance to I:C but not to NDV.

Cell treatment with interferon. Paucker and Boxaca (1) reported that interferon mediated the resistance of L cells to the production of interferon by NDV. In order to determine the role of interferon in the development of tolerance to NDV and I:C in human fibroblasts, the studies shown in Fig. 1 were repeated using cell treatment with interferon in place of the initial incubation with I:C. The dose of interferon employed in these studies was similar to that produced in response to treatment with I:C. The results are shown in Fig. 2, expressed in percentage of the interferon response with I:C and NDV (400 and 1900 units/4 ml, respectively) in controls not previously treated with interferon. It is seen that interferon reduced the amounts of interferon produced by NDV but not by I:C. Therefore, it appears that interferon, produced during treatment with I:C,

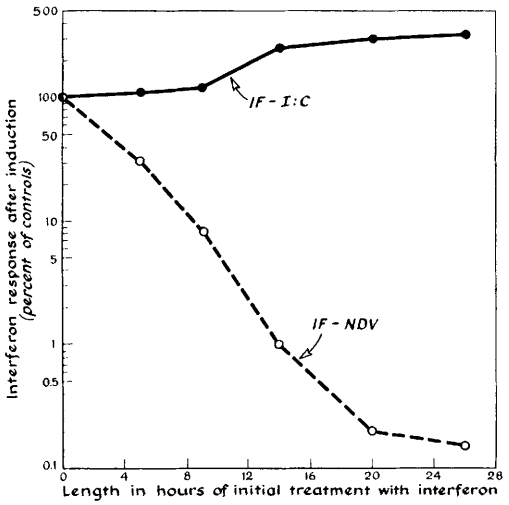

FIG. 2. Effect of cell treatment with interferon on the subsequent stimulation of interferon by NDV and I:C. The same experimental design was employed as in Fig. 1, except that interferon (400 units in 4 ml) was used in place of the initial treatment with I:C.

rendered the cells resistant to the induction of interferon by NDV (Fig. 1). However, a different mechanism appeared responsible for the tolerance of human fibroblasts to a second dose of I:C.

Youngner and Hallum (8) found that treatment of L cells with interferon inhibited the subsequent stimulation of interferon production by I:C and DEAE–dextran. This finding is in contrast to our results in human fibroblasts where interferon had an enhancing effect (Fig. 2). It raises the possibility that the mechanism of interferon production by I:C in L cells differs from that in human fibroblasts. It is possible that interferon is released from a pre-formed pool in human fibroblasts but synthesized *de novo* in L cells. There are fundamental differences between L cells and other cell systems in regard to some characteristics of interferon stimulation by I:C. As first noted by Dianzani *et al.* (9), L cells are not able to produce interferon unless DEAE–dextran is also present. Full yields of interferon are produced after 5.5 hr in rabbit kidney cells (10) and 8 hr in human fibroblasts (4), while 18 hr are required with I:C and DEAE–dextran in L cells.

Repeated interferon induction in actinomycin D-treated cells. Previous studies (4, 6) demonstrated that pretreatment of human fibroblasts with a moderate dose of actinomycin D (0.3 μg/ml) did not diminish the yield of interferon induced by I:C. This finding enabled us to investigate whether the development of I:C-tolerance was more sensitive to inhibition of RNA synthesis than the induction of interferon by I:C. It appeared that cells treated with actinomycin D before exposure to I:C did not develop I:C-tolerance but remained sensitive to repeated stimulation of interferon by I:C (Table I).

Effect of brief treatment with I:C on development of I:C-tolerance. Human fibroblasts produce high levels of interferon after only a brief exposure to I:C followed by incubation with TCM at 37° (3). Table II shows that cell monolayers produced 320 units of interferon after treatment with I:C for only 1 hr followed by washing and additional incubation with TCM for 8 hr. However, it was found that I:C-tolerance did not develop under these conditions (Table II).

A preliminary experiment indicated that cell extracts of human fibroblast monolayers that were treated with I:C (50 μg/ml, 9 hr) did not degrade 2 μg/ml of I:C-^{3}H (0.01 μCi/μg) more rapidly than extracts of cell monolayers treated with MEM. Assays were performed both at pH 7.2 and pH 5.0. In view of the very small amounts of I:C that enter the cell in comparison to the total amount of I:C added to cells (3), it appears quite possible that such an assay is too crude to measure an increase in the rate of intracellular degradation of I:C after prolonged exposure of cells to I:C.

Discussion. It appears that two different mechanisms account for the occurrence of NDV-tolerance and I:C-tolerance. In accordance with the results in Figs. 1 and 2, and of others (1), we propose the following explanation for the hyporeactivity of cells, after treatment with I:C, to the stimulation of interferon by virus (NDV-tolerance). I:C produces in cells a state of antiviral resistance, mediated by interferon. Upon infection of such cells with NDV, the translation of early viral functions is likely to be blocked (11). Consequently, the viral function re-

TABLE I. Repeated Stimulation of Interferon by I:C in Actinomycin D-pretreated Cells.[a]

Length of first treatment with I:C (or TCM) (hr)	Interferon in cell supernatant fluid after second induction (units/4 ml)		
	Control I[b] (I:C–TCM)	Control II[c] (TCM–I:C)	Experimental (I:C–I:C)
Actinomycin pretreated			
9	165	100	300
14	128	280	460
20	120	260	360
No actinomycin			
9	30	300	42
14	25	300	40
20	12	300	112

[a] Human fibroblasts were treated for varying times at 37° with I:C (or TCM) as shown. The cell monolayers were then washed and additionally incubated for 9 hr with either I:C or TCM, as indicated. After this time the cell supernatant fluids were assayed for interferon.

[b] Controls receiving TCM instead of the second dose of I:C.

[c] Controls receiving TCM instead of the first dose of I:C.

sponsible for the initiation of interferon production is not expressed. In agreement with this model, we found that the characteristic inhibition by NDV of host RNA and protein synthesis (4, 12) did not occur in I:C-treated human fibroblasts (our unpublished observation). Furthermore, Chany and Vignal (13) recently reported a mutant cell line that could produce interferon but had lost sensitivity to the action of interferon. It was found that hyporeactivity to repeated stimulation of interferon by NDV did not occur in this cell line (13).

Vilcek and associates (10, 14) demonstrated a prolonged and elevated interferon response in rabbit kidney cells when treated with a high dose of actinomycin D after interferon stimulation by I:C had commenced. The results summarized in Table I suggest that this observation might be related to the inhibitory effect of actinomycin D on the development of I:C-tolerance. In the absence of actinomycin D, maximum I:C-tolerance was observed around the time when human fibroblasts normally terminate the interferon response with I:C (Fig. 1).

The findings presented in this report do not provide conclusive evidence for a mechanism of I:C-tolerance. However, Table II suggests that I:C-tolerance is not specifically due to the production of interferon but due to other effects or prolonged exposure of cells to I:C. The reason for the reduction of I:C-tolerance after initial treatment of cells with I:C for over 20 hr (Fig. 1) is not known, but one of several possibilities is the enhancing

TABLE II. Effect of Brief Treatment with I:C on the Development of I:C-Tolerance.[a]

Primary treatment with I:C at 37° (hr)	Primary interferon response (units/4 ml)	Interferon response after repeated stimulation with I:C		
		Control I (I:C–TCM)	Control II (TCM–I:C)	Experimental (I:C–I:C)
9	530	35	200	45
1—followed by TCM (8 hr)	320	40	230	220

[a] After the indicated primary treatment with I:C, human fibroblasts were washed and additionally incubated for 9 hr at 37° with either I:C or TCM as shown. The controls employed are described in Table I.

effect of prolonged cell treatment with interferon on interferon production with I:C (Fig. 2).

Our earlier studies in human fibroblasts suggested that the interferon response with I:C is terminated after 8 hr although the vast majority of the initial input I:C remains in the cell supernatant fluid in undegraded form (3, 4). The fraction of I:C that is taken up by cells, however, undergoes predominantly degradation and incorporation into cellular RNA (3). We propose that I:C-tolerance may occur due to an increase of intracellular ribonuclease in response to prolonged uptake of I:C, preventing prolonged stimulation of interferon by prematurely degrading the polynucleotide. Although such a model of I:C tolerance is consistent with the available evidence, its verification must await experimental proof by an approach more sensitive than the preliminary experiment presented in this report.

After these studies were completed, we learned that Vilcek (personal communication, 1970) has independently demonstrated differences between I:C- and NDV-tolerance in rabbit kidney cells after exposure to I:C. Although somewhat different approaches were used, the results were in agreement with the findings presented in Figs. 1 and 2.

Summary. Human fibroblasts treated with the synthetic interferon-stimulating polymer polyinosinate:polycytidylate (I:C) became resistant (or "tolerant") to a subsequent stimulation of interferon by either I:C or Newcastle disease virus (NDV). Tolerance to I:C reached a maximum around the time of termination of the first interferon response. After this time the cells gradually recovered some sensitivity to a second stimulation of interferon by I:C. In contrast, tolerance to NDV persisted and increased after termination of the primary interferon response by I:C. Treatment of human fibroblasts with interferon inhibited the subsequent production of interferon by NDV but not by I:C. When human fibroblasts were pretreated with a moderate dose of actinomycin D, tolerance to I:C was not observed. Furthermore, tolerance to I:C was significantly diminished when cells were only briefly exposed to I:C, although high levels of interferon were produced by this treatment. A model is proposed by which cells develop resistance to repeated stimulation of interferon.

1. Paucker, K., and Boxaca, M., Bacteriol Rev. **31**, 145 (1967).

2. Field, A. K., Tytell, A. A., Lampson, G. P., and Hilleman, M. R., Proc. Nat. Acad. Sci. U.S.A. **58**, 1004 (1967).

3. Bausek, G. H., and Merigan, T. C., Virology **39**, 491 (1969).

4. Bausek, G. H., and Merigan, T. C., Proc. Soc. Exp. Biol. Med. **133**, 982 (1970).

5. Merigan, T. C., Gregory, D. F., and Petralli, J. K., Virology **29**, 515 (1966).

6. Finkelstein, M. S., Bausek, G. H., and Merigan, T. C., Science **161**, 465 (1968).

7. Merigan, T. C., Winget, C. A., and Dixon, C. B., J. Mol. Biol. **13**, 679 (1965).

8. Youngner, J. S., and Hallum, J. V., Virology **37**, 473 (1969).

9. Dianzani, F., Cantagalli, S., Gagnoni, S., and Rita, G., Proc. Soc. Exp. Biol. Med. **128**, 708 (1968).

10. Vilcek, J., Rossman, T. G., and Varacalli, F., Nature **223**, 682 (1969).

11. Friedman, R. M., J. Gen. Physiol. in press (1970).

12. Wilson, D. E., J. Virol. **2**, 1 (1968).

13. Chany, C., and Vignal, M., C. R. H. Acad. Sci. Ser. D **267**, 1798 (1968).

14. Vilcek, J., Ann. N. Y. Acad. Sci., in press (1970).

Received March 6, 1970. P.S.E.B.M., 1970, Vol. 134.