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Specificity of Interferon-induced Enhancement of Toxicity for Double-stranded Ribonucleic Acids

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SUMMARY

Interferon-treated cells exhibited an increased susceptibility to the toxicity of several natural and synthetic double-stranded RNA molecules and to vaccinia virus, provided the latter was able to undergo replicative events. However, interferon-treated cells exhibited no enhanced susceptibility to several other toxic materials or to vaccinia virus that was unable to replicate. These findings suggest that interferon-treated cells exhibit a specific enhanced susceptibility to the toxicity of double-stranded RNA molecules. Since neither RNA nor protein synthesis was required for toxicity to occur in interferon-treated cells exposed to polyriboinosinic-polyribocytidylic acid, it appears that the double-stranded RNA configuration is directly lytic for interferon-treated cells.

INTRODUCTION

We have recently reported that interferon-treated cells are much more sensitive to the toxicity of the synthetic double-stranded (ds) RNA polyriboinosinic.polyribocytidylic acid (poly I.poly C) than are normal cells (Stewart *et al.* 1972*a*). This observation led us to question whether interferon-treated cells might be more susceptible to toxic substances in general. Indeed, Joklik & Merigan (1966), Horak, Jungwirth & Bodo (1971) and Jungwirth *et al.* (1972) have observed an exaggeration of the disintegration of interferon-treated L cells following infection with vaccinia virus. In this communication we report experiments designed to determine whether interferon-treated cells are generally more sensitive to all classes of toxic substances or whether this enhanced susceptibility of interferon-treated cells is specific for a particular class of toxic materials.

METHODS

Cells and viruses. Monolayer cultures for use in experiments were prepared by inoculating 60 mm plastic plates with approximately 10^6 L929 cells, diploid human embryonic skin (HES) fibroblasts, or HeLa cells in 3 ml of growth medium (GM) consisting of Eagle's minimal essential medium supplemented with 10 % bovine serum and antibiotics. Monolayers were near confluency after overnight incubation in 5 % CO_2 atmosphere at 37 °C, at which time they were used. A commercial calf lymph vaccine of vaccinia virus, received from the Vaccines Office in Brussels, was passaged in chorioallantoic membranes of 11-day embryonated chicken eggs to prepare a stock suspension. A stock of vesicular stomatitis virus (VSV), Indiana serotype, was prepared by propagation of the virus in BSC-1 cell cultures.

Interferons. Mouse interferon was prepared in L929 cell cultures inoculated with MM virus as previously described (Stewart, Gosser & Lockart, 1971*a*). Human interferon was prepared in HES cultures inoculated with poly I.poly C as described (Stewart *et al.* 1972*a*).

Toxic substances. Polyriboinosinic acid (poly I) and polyribocytidylic acid (poly C) were purchased from P-L Biochemicals, Inc., Milwaukee, Wisconsin, or from Miles Laboratories, Elkhart, Indiana. Poly I.poly C was prepared by annealing the individual homopolymers in equal amounts (1 mg/ml) in 0·15 M-NaCl at 45 °C for 1 h and was stored frozen at −20 °C. Polyriboadenylic acid.polyribouridylic acid (poly A.poly U) was prepared from the homopolymers obtained from Miles Laboratories as previously described (De Clercq & De Somer, 1972). The heteropolymeric duplex of deoxyriboadenylic acid and deoxyribothymidylic acid, poly[d(A–T)].poly[d(A–T)], was obtained from Miles Laboratories. Double-stranded RNA from mycophage of *Penicillium* BRL-5907 was obtained from D. N. Planterose, Beecham Research Laboratories, Betchworth, Surrey, England. Double-stranded RNA from bacteriophage f2 (Doskocil *et al.* 1971) was obtained from L. Borecky, Institute of Virology, Bratislava, Czechoslovakia. Calf thymus DNA, type V, and cycloheximide were obtained from the Sigma Chemical Co., St Louis, Missouri. Tilorone-HCl was generously provided by R. F. Krueger, William S. Merrell Co., Cincinnati, Ohio. Chlorite-oxidized oxyamylose (COAM) was synthesized by P. Claes of our Institute (Claes *et al.* 1970). *Salmonella enteritidis* endotoxin was obtained from Difco Laboratories, Detroit, Michigan. Actinomycin D was obtained from Schwarz-Mann, Orangeburg, N.Y. Diethylaminoethyl-dextran (DEAE-dextran) was obtained from Pharmacia, Uppsala, Sweden. Venom from *Micrurus fulvius* (MP no. 37211), *Crotalus terrificus* (MP no. 42894) and *Crotalus adamanteus* (MP no 51369) were kindly provided by C. P. Hegarty, Wyeth Laboratories, Inc., Marietta, Pennsylvania. Diphtheria toxin, a crude culture extract, was obtained from A. Billiau of our Institute. Purified cholera toxin, lot 1071, was prepared under contract for the National Institute of Allergy and Infectious Diseases by R. A. Finkelstein, Ph.D., The University of Texas Southwestern Medical School, Dallas, Texas (Finkelstein & La Spalluto, 1970).

RESULTS

Comparisons of the toxicities of various substances for normal and interferon-treated cells

Since interferon-treated cells were more susceptible than normal cells to the toxicity of poly I.poly C (Stewart *et al.* 1972*a*) it seemed possible that interferon might itself be somewhat damaging to cells, in which case interferon treatment might be expected to non-specifically increase the susceptibility of cells to any toxic materials. We therefore compared the sensitivities of normal and interferon-treated cells to a wide variety of toxic substances. In these experiments, interferon treatment consisted of incubating cell cultures overnight with 2 ml of GM containing 100 units of homologous interferon/ml. It should be noted that this interferon treatment is one that primes subsequent interferon production by poly I.poly C (Stewart *et al.* 1972*a*). Cultures were then washed 3 times with serum-free GM and were incubated for 1 h with 1 ml of the test material of the indicated concentrations in serum-free GM. Cultures were then washed once and further incubated with 2 ml of serum-free GM. Toxicity was recorded as the amount of c.p.e observed. Comparisons were thus made of the amount of toxic substance required to give c.p.e. in normal and interferon-treated cultures. Since not all the materials tested were toxic for L929 cells, it was necessary to assay some substances for toxicity in human cells which also exhibited

enhanced sensitivity to poly I.poly C after interferon treatment (Stewart *et al.* 1972*a*; W. E. Stewart, E. De Clercq & P. De Somer, manuscript in preparation). As shown in Table 1, poly I.poly C, which was not detectably toxic to normal L929 cells at 50 μg, was toxic to cultures of interferon-treated L929 cells at only 2 μg; poly A.poly U was not toxic to normal cells at 50 μg but was toxic to interferon-treated cells, though less so than was poly I.poly C; mycophage ds RNA and bacteriophage ds RNA were likewise toxic to interferon-treated cells, though not to normal cells. De Clercq & De Somer (1971, 1972) have reported that separate administration of poly I followed by poly C gives even better induction of interferon than the paired polymers applied together, and we found that the separately administered polymers were even more toxic than poly I.poly C, provided that poly I was applied first, followed by poly C. However, if the sequence of application was reversed, no toxicity was observed in either normal or interferon-treated cells at the doses employed. These results are in good agreement with the findings of De Clercq & De Somer (1972), showing that poly I followed by poly C induced more antivirus activity than the double-stranded RNA itself but that poly C followed by poly I failed to exhibit enhanced activity. These authors suggested that the failure of poly C to 'prime' the activity of poly I resulted from the rapid degradation of poly C by nucleases. Vaccinia virus was toxic for interferon-treated L929 cells at high multiplicities. This toxic degeneration of interferon-treated L cells infected with vaccinia virus, as has been described previously (Joklik & Merigan, 1966; Horak *et al.* 1971; Jungwirth *et al.* 1972), developed prior to, and was distinguishable from, the virus-induced c.p.e., which was characterized by rounding of cells. The c.p.e. resulting from enhanced toxicity in interferon-treated cells exposed to poly I.poly C, poly A.poly U, mycophage ds RNA, bacteriophage ds RNA, poly I followed by poly C, or vaccinia virus was characteristic and had completely developed by 5 h after removal of the toxic substance (6 h after exposure), with no significant increase in the amount of c.p.e. beyond this time. We shall henceforth refer to the early degeneration of interferon-treated cells as 'lysis' to distinguish this effect from the normal c.p.e. of vaccinia virus and the toxicities resulting in both normal and interferon-treated cells exposed to some of the other toxic materials, which usually developed much later than the 'lysis'. COAM, endotoxin, actinomycin D, cycloheximide, DEAE-dextran, snake venoms, tilorone-HCl, calf thymus DNA, cholera toxin and diphtheria toxin were all toxic to interferon-treated cells, but were toxic at the same concentrations to normal cells, and the resulting toxicities in each case were distinct from the lytic degeneration seen in the interferon-treated cells exposed to the ds RNAs or vaccinia virus. Poly[d(A–T)].poly[d(A–T)] was not toxic to either normal or interferon-treated cells at the concentrations tested. It should be mentioned that our data with diphtheria toxin in HeLa cells do not appear to agree with the findings of Moehring, Moehring & Stinebring (1971), who reported being able to repeat the finding of Yabrov (1967) that interferon-treated cells were protected from the toxic effects of diphtheria toxin. Though the reasons for this discrepancy are not apparent, it is possible that the range of doses we employed (tenfold increments) were not sufficiently small to enable us to detect slight amounts of protection. This interpretation is enhanced by the fact that in Yabrov's experiments the difference in toxin concentrations ranged from 1·5- to 5-fold and in the experiments of Moehring *et al.* (1971) the protection was slight.

These data suggest that the increased susceptibility of interferon-treated cells is specific for the toxicity of the ds RNA configuration and for vaccinia virus.

Table 1. *Comparison of the sensitivities of normal and interferon-treated cells to toxic substances*

Toxic substance*	Dose	Toxicity†	
		Normal	Interferon-treated
Poly I. poly C	2 μg	−	++
	10 μg	−	+++
	50 μg	−	++++
Poly A. poly U	2 μg	−	−
	10 μg	−	±
	50 μg	−	+
Mycophage ds-RNA	2 μg	−	+
	10 μg	−	++
	50 μg	−	+++
Bacteriophage ds-RNA	2 μg	−	±
	10 μg	−	+
	50 μg	−	++
Poly I followed by poly C	0·5 μg each	−	+++
	2·5 μg each	−	++++
	12·5 μg each	−	++++
Vaccinia virus	2×10^6 p.f.u.	−	−
	1×10^7 p.f.u.	−	±
	5×10^7 p.f.u.	−	++
Poly C followed by poly I	0·5 μg each	−	−
	2·5 μg each	−	−
	12·5 μg each	−	−
Poly I	10 μg	−	−
	50 μg	−	−
	250 μg	−	−
Poly C	10 μg	−	−
	50 μg	−	−
	250 μg	−	−
Poly [d(A-T)].poly [d(A-T)]	4 μg	−	−
	20 μg	−	−
	100 μg	−	−
Calf thymus DNA	10 μg	−	−
	100 μg	−	−
	1000 μg	±	±
Tilorone-HCl	1 μg	−	−
	10 μg	+	+
	100 μg	++++	++++
Endotoxin	1 μg	−	−
	10 μg	+	+
	100 μg	++	++
Cycloheximide	20 μg	−	−
	100 μg	+	+
	500 μg	++	++
Actinomycin D	0·5 μg	±	±
	5·0 μg	++	++
	50·0 μg	+++	+++
COAM	10 μg	−	−
	100 μg	−	−
	1000 μg	+	+

Table 1 (*cont.*)

Toxic substance*	Dose	Toxicity†	
		Normal	Interferon-treated
DEAE-dextran	20 μg	−	−
	100 μg	+	+
	500 μg	+++	+++
Crotalus adamanteus venom	0·05 μg	−	−
	0·5 μg	−	−
	5·0 μg	+	+
Crotalus terrificus venom	0·5 μg	−	−
	5·0 μg	−	−
	50·0 μg	+	+
Micrurus fulvius venom	0·5 μg	−	−
	5·0 μg	−	−
	50·0 μg	+	+
Diphtheria toxin	0·1 guinea-pig	±	±
	1·0 LD_{50}	++	++
	10·0 LD_{50}	++++	++++
Cholera toxin	0·1 μg	−	−
	1·0 μg	+	+
	10·0 μg	+++	+++

* All determinations were performed on L929 cell cultures except those for diphtheria and cholera toxins, which were performed on HeLa and HES cultures, respectively.

† Toxicity recorded as amount of c.p.e.: −, none; ±, cultures with floating cells and debris but no disruption of monolayers; +, 0 to 25 %; ++, 25 to 75 %; +++, 75 to 95 %; ++++, complete destruction of monolayers. C.p.e. readings were made at 6 h after exposure to poly I.poly C, poly A.poly U, mycophage ds RNA, bacteriophage ds RNA and after administration of poly C subsequent to poly I; c.p.e. readings for vaccinia virus infected cultures were made at 4 h after infection; all other c.p.e. readings were made 24 h after addition of test material. Determinations with poly I.poly C, poly A.poly U, mycophage ds RNA and bacteriophage ds RNA were performed simultaneously so comparisons of relative toxicities could be made.

Metabolic requirements for enhanced toxicity of vaccinia virus in interferon-treated L929 cells

Joklik & Merigan (1966) reported that the disintegration of interferon-treated L cells began about 3 h after infection with vaccinia virus and that even at input multiplicities as low as 50 p.f.u./cell practically all the interferon-treated cells had been converted to 'ghosts' by 6 h after infection, while infected cells not previously exposed to interferon were practically all intact. Subsequently Horak *et al.* (1971) presented evidence that a virus gene product must be synthesized for the lysis of vaccinia virus infected interferon-treated cells. However, in the L cells employed by the latter authors, lysis occurred somewhat later, between 4 and 8 h after infection, and these authors state that in L cells from another source the phenomenon occurs at an even later time. It seemed possible therefore that the same product, virus or virus-induced, might not have been responsible for the lysis observed in these different systems and that the input virus particle itself could have induced the lysis of interferon-treated cells reported by the former authors. Since the kinetics of lysis of the interferon-treated L cells infected with vaccinia virus in our studies was similar to that described by Joklik & Merigan (1966), beginning at about 3 h after infection with a multiplicity of 50, with practically complete lysis developed by 6 h after infection, we performed experiments to determine whether the input virus could account for the lysis of interferon-

Table 2. *Metabolic requirements for enhanced toxicity of vaccinia virus in interferon-treated L929 cells*

Inoculum*	Toxicity†	
	Normal	Interferon-treated
Vaccinia virus	−	++
Vaccinia virus + cycloheximide	−	−
Vaccinia virus + actinomycin D	−	−
Heat-inactivated vaccinia virus	−	−

* Cultures were incubated for 1 h at 37 °C with 1 ml of serum-free GM containing either vaccinia virus (m.o.i. 50), vaccinia virus + 5 μg actinomycin D, vaccinia virus + 100 μg cycloheximide, or vaccinia virus that had been incubated 1 h at 60 °C. Cultures were then washed once and incubated further with 2 ml serum-free GM. Inocula containing cycloheximide or actinomycin D were replaced with serum-free GM containing 100 μg cycloheximide/ml or 5 μg actinomycin D/ml, respectively.

† Toxicity recorded as amount of c.p.e. was recorded at 4 h after infection.

treated cells or whether a virus gene product must be synthesized in this system to produce lysis, such as was the case in the system employed by Horak *et al.* (1971).

Cultures of normal and interferon-treated L929 cells were infected with either live vaccinia virus at a multiplicity of 50, or a similar amount of virus which had been inactivated by heating for 1 h at 60 °C. Some cultures were treated with either cycloheximide (100 μg/ml) or actinomycin D (5 μg/ml). Lysis, recorded at 4 h after infection, was prior to and distinguishable from the virus-induced cell-rounding that began developing in untreated cells at about 4 to 6 h after infection. As shown in Table 2, lysis was observed only if virus gene products could be synthesized. No lysis of interferon-treated cells was observed when either actinomycin D or cycloheximide were incorporated into the medium, nor could heat-inactivated virus induce cell lysis. These results confirm the interpretation of Horak *et al.* (1971) that a virus gene product, not the DNA of the infecting virus itself, is responsible for the lysis of interferon-treated L cells.

These data suggest that the increased susceptibility of interferon-treated cells is specific for the toxicity of ds RNA configurations and for a product of vaccinia virus replication.

Lack of metabolic requirements for enhanced toxicity in interferon-treated L929 cells exposed to poly I.poly C

The finding that the different types of ds RNA molecules are toxic for interferon-treated cells, together with the observation that the degree of lysis induced in interferon-treated cells by these molecules correlates with the relative abilities of the polynucleotides to induce interferon production in cells (i.e. poly I followed by poly C > poly I.poly C > poly A.poly U > poly[d(A–T)].poly[d(A–T)]; Colby *et al.* 1971; De Clercq & De Somer, 1971, 1972; Stewart *et al.* 1971*a*, 1972*a*; De Clercq, Wells & Merigan, 1972; W. E. Stewart, E. De Clercq & P. De Somer, manuscript in preparation), suggested that the production of interferon in interferon-treated cells might be lethal. Therefore, experiments were performed to determine the effect of protein synthesis inhibition (and, therefore, inhibition of interferon production) on the toxicity of poly I.poly C for interferon-treated L929 cells. As shown in Table 3, the toxicity of poly I.poly C for interferon-treated cells was even more pronounced in the presence of the protein synthesis inhibitor cycloheximide. Next we explored the possibility that the induction of interferon messenger RNA in interferon-treated cells might be the lethal event. Experiments were performed to determine the effect of inhibition of DNA-

Table 3. *Metabolic requirements for enhanced toxicity of Polyriboinosinic.polyribocytidylic acid in interferon-treated L929 cells*

Inoculum*	Toxicity†	
	Normal	Interferon-treated
Poly I.poly C	–	+++
Poly I.poly C+cycloheximide	–	++++
Cycloheximide	–	–
Poly I.poly C+actinomycin D	–	++++
Actinomycin D	–	–

* Monolayer cultures of L929 cells treated with interferon, as in Table 1, were incubated for 1 h at 37 °C with 1 ml serum-free GM containing 10 μg poly I.poly C, 10 μg of poly I.poly C+100 μg cycloheximide or 5 μg of actinomycin D, 100 μg cycloheximide alone, or 5 μg actinomycin D alone. Cultures were then washed once and incubated further with 2 ml serum-free GM. Inocula containing cycloheximide or actinomycin D were replaced with serum-free GM containing 100 μg cycloheximide or 5 μg actinomycin D, respectively.

† Toxicity recorded as amount of c.p.e. was recorded at 6 h after inoculation.

dependent RNA synthesis on the toxicity of poly I.poly C for interferon-treated L929 cells. As shown in Table 3, the toxicity of poly I.poly C for interferon-treated cells was even more pronounced in the presence of actinomycin D.

These data, showing that neither RNA nor protein synthesis is required for poly I.poly C to destroy interferon-treated cells, suggest that the ds RNA configuration is directly lytic for interferon-treated cells.

DISCUSSION

These studies show that our previous observation that interferon-treated cells were more susceptible to the toxicity of poly I.poly C (Stewart *et al.* 1972*a*) cannot be attributed to a nonspecific damage of cells by interferon, thereby making them more sensitive to any toxic material. Of all the materials tested, with the apparent exception of vaccinia virus, only those possessing a ds-RNA configuration were found to be more toxic for interferon-treated cells than for normal cells. The fact that vaccinia virus was unable to induce lysis of interferon-treated cells in the presence of RNA or protein synthesis inhibitors, while poly I.poly C was able to do so under similar conditions, suggests, in confirmation of the interpretation of Horak *et al.* (1971), that a virus gene product, not the virus itself, is responsible for the lysis of interferon-treated L cells. Since Colby & Duesberg (1969) have reported the presence of ds-RNA in cells infected with vaccinia virus, it is tempting to speculate that the enhanced susceptibility of interferon-treated cells is, indeed, specific for ds-RNA configurations.

Recent experiments by Marcus *et al.* (1971) suggest that transcription of virus RNA is a site of antivirus action of interferon in chick cells infected with VSV. This observation has been extended to vaccinia virus-infected chick cells (Bialy & Colby, 1972) and to VSV-infected human cells (E. Manders, J. Tilles & A. Huang, personal communication, 1972). More recently, however, Metz & Esteban (1972) have presented evidence that interferon works at the level of translation rather than transcription since they were able to detect virus RNA synthesis in interferon-treated L cells infected with vaccinia virus but found that protein synthesis ceased shortly after infection. Our interpretation that vaccinia virus-induced lysis of interferon-treated L cells is attributable to a virus gene product (i.e. a ds-RNA configuration) implies that such virus products can be made in interferon-treated

L cells infected with vaccinia virus. If this is the case, and if interferon does indeed act at the level of transcription in some systems, it would appear that either interferon inhibits the same virus by different mechanisms in different cells or, alternatively, that vaccinia virus replicative processes are actually not inhibited in mouse L cells treated with homologous interferon. The failure of vaccinia virus to form infective virus particles in interferon-treated L cells could then be a consequence of cell lysis, as proposed by Joklik & Merigan (1966), rather than due to interferon-induced antivirus activity. If vaccinia virus were inhibited at the level of transcription in interferon-treated L cells, we would have to rationalize that some virus replicative events occurred in the interferon-treated cells simply because not enough interferon has been used to give complete inhibition of virus replication and that some ds-RNA could therefore be produced to lyse the interferon-treated cells. However, recent analysis of virus specific functions in interferon-treated L cells and chick cells infected with vaccinia virus reveal that, while formation of virus particles was almost completely inhibited in both cells, early virus-specific syntheses were initially inhibited only in chick cells (Jungwirth *et al.* 1972). These findings support the interpretation of Joklik & Merigan (1966) that the failure of interferon-treated L cells to replicate vaccinia virus is due to cell destruction before virus can be produced. This implies that vaccinia virus replicative processes are not actually sensitive to the interferon-induced antivirus activity in L cells but are sensitive, at an early stage, to the antivirus activity induced in chick cells by homologous interferon. Such host-dependent variations in sensitivities of viruses to the antivirus activities induced by interferon have been suggested (Stewart, Scott & Sulkin, 1969; Hallum, Thacore & Youngner, 1970; Stewart & Lockart, 1970). Alternatively, interferons could inhibit virus by different mechanisms in different types of cells, for, as has been repeatedly pointed out (Stewart *et al.* 1969; Hallum *et al.* 1970; Stewart & Lockart, 1970; Marcus *et al.* 1971; Bialy & Colby, 1972), there is no reason for suggesting that the interferon system consists of a single mechanism of action.

The fact that interferon-treated cells are lysed by a particular molecular structure that is not lytic for normal cells, whether this structure is exclusively ds-RNA or not, shows that not only are interferon-treated cells altered in their abilities to replicate viruses but that interferon treatment alters cells in some manner unrelated to antivirus activity. Similar conclusions have been arrived at from studies on the ability of interferon to enhance interferon production in cells exposed to various interferon inducers (Stewart *et al.* 1971*a*, *b*, 1972*b*) and the ability of interferon to inhibit the growth of cells (Paucker, Cantell & Henle, 1962; Gresser *et al.* 1970*a*, *b*, 1971; Lindahl-Magnusson, Leary & Gresser, 1971; Macieira-Coelho *et al.* 1971; Lee, O'Shaughnessy & Rozee, 1972). Further studies will, hopefully, provide a clearer understanding of the nature of the alteration of cells by interferon that results in their fragility to ds-RNA molecules and the nature of the interaction of ds-RNA with interferon-treated cells that results in lysis.

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