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Cellular Alteration by Interferon: a Virus-Free System for Assaying Interferon

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A system is described for assaying mouse interferon without using a viral "challenge" agent. Interferon-treated L cells were destroyed by exposure to polyriboinosinic·polyribocytidylic acid [poly(I)·poly(C)], and the amount of destruction was dependent on both the concentration of interferon to which the cells were exposed and the amount of poly(I)·poly(C) used as the "challenge" material. If the amount of poly(I)·poly(C) was constant, the concentration of interferon could be determined by quantitating cell destruction 6 hr after addition of the double-stranded ribonucleic acid. In addition to eliminating the necessity for employing infectious virus for interferon assays, this system has the advantages of being quicker, easier, and more sensitive than other interferon assays. The sensitivity of the assay is related directly to the amount of poly(I)·poly(C) applied to the cells, with each fivefold increase of poly(I)·poly(C) giving about a fivefold increase of sensitivity.

The continuing quest for a better interferon assay, as evidenced by the numerous publications describing techniques for assaying interferons (1-8, 12, 14, 19, 21, 24, 25, 31, 32, 34, 35), is proof of the dissatisfaction of investigators with the available methods. All of these assays have been based on the ability of interferon to interrupt the normal replicative processes of viruses, as determined by various direct or indirect methods. These procedures for quantitating interferons differ in complexity and sensitivity, and in the amount of time required to get results, but each has at least one of the disadvantages of being insensitive, time-consuming, or laborious. An ideal interferon assay would measure the effect of interferon on cells quickly while minimizing technical complexity and maximizing sensitivity.

The finding (28) that interferon-treated cells were more responsive than normal cells to induction of interferon by the synthetic double-stranded ribonucleic acid (RNA) polyriboinosinic·polyribocytidylic acid [poly(I)·poly(C)] led us to determine that interferon-treated cells were more susceptible than normal cells to the toxicity of poly(I)·poly(C) (26) and other double-stranded RNA molecules (Stewart, De Clercq, and De Somer, *in preparation*). Inasmuch as the amount of cell destruction caused by a given amount of poly(I)·poly(C) was dependent on the concentration of interferon to which cells had been exposed, it seemed possible to use this non-antiviral measurement of cellular alteration by interferon to assay interferon preparations, thus eliminating the necessity of dealing with a replicative agent. The experiments described here were directed toward that end.

MATERIALS AND METHODS

Cells and virus. L-929 cells and Lpa cells, the source of which has been described elsewhere (28), were grown in monolayers in Eagle minimal essential medium (MEM) supplemented with 10% bovine serum (BS) and antibiotics in an atmosphere of 5% CO_2 at 37 C. Cultures for experiments were prepared by inoculating 60-mm plastic tissue culture plates (Falcon Plastics) with 3 ml of MEM plus 10% BS containing approximately 10^6 cells. Cultures were then incubated at 37 C for 6 hr, at which time monolayers were nearly confluent and were used. Vesicular stomatitis virus (VSV) strain Indiana was propagated in BSC-1 cells.

Reagents. Poly(I)·poly(C) was prepared as described elsewhere (Stewart et al., *in preparation*) from homopolymers purchased from either P-L Biochemicals, Inc., Milwaukee, Wis., or Miles Laboratories, Elkhart, Ind.

Crude L cell interferon (specific activity, about 10^4 units/mg of protein) was prepared by inoculating monolayer cultures of L-929 cells with Newcastle disease virus (NDV) at a multiplicity of infection of 10. Partially purified L cell interferons were prepared and purified as described (28) to give preparations with a specific activity of approximately 10^5 and 5×10^6 units/mg of protein. The latter preparation was kindly supplied to us by E. Knight, DuPont Co., Wilmington, Del. Mouse serum interferon was prepared by inoculating weanling NMRI mice with NDV (4×10^7 plaque-forming units [PFU]/mouse) and collecting blood 16 hr later. Interferon titers are

expressed as the reciprocal of the dilutions that depress the number of plaques of VSV by 50% (PDD_{50}-VSV) on L-929 cells. One PDD_{50}-VSV unit is equivalent to approximately 2 units of international mouse reference interferon.

Interferon plaque-reduction assays. Monolayer cultures of the indicated cells were incubated overnight with 2 ml of serial 0.5 $\log_{10}$ dilutions of interferon preparations, two cultures/dilution. Media were then aspirated, and 0.5 ml of VSV suspension containing 100 to 200 PFU was added. After 1 hr of adsorption at 37 C, inocula were removed, and plates were overlaid with 3 ml of medium containing 1% agar, 2% BS, MEM, and antibiotics, adjusted to *p*H 7.2 to 7.4 with $NaHCO_3$. Plates were then incubated at 37 C for 48 hr, at which time plaques were counted and 50% plaque-reduction end points were estimated by intersection of an interpolated line from plotted points.

RESULTS

Assay of interferon by determining altered susceptibility of cells to the toxicity of poly(I)·poly(C). We previously reported that interferon-treated L-929 cells are destroyed when exposed to poly(I)·poly(C) and that the degree of cellular destruction is related to both the concentration of interferon with which cells were incubated and the level of poly(I)·poly(C) applied (26). Experiments were therefore performed to determine the amount of interferon required to alter cells sufficiently so that a dose of 10 μg of poly(I)·poly(C) would produce a clearly discernible cytopathic effect (CPE). These experiments were modified wherever possible to eliminate manipulations. Monolayer cultures of L-929 cells were incubated overnight at 37 C with 2 ml of serial 0.5 $\log_{10}$ dilutions of various mouse interferon preparations in MEM plus 10% BS. These inocula were then aspirated, and half of the cultures were challenged with VSV, overlaid, and incubated at 37 C for 48 hr, at which time plaques were counted. Media from the other cultures were replaced with 1 ml of serum-free MEM containing 10 μg of poly(I)·poly(C), and these cultures were incubated at 37 C until CPE was fully developed. Initially, CPE was recorded at 30 times magnification at 2, 4, 6, and 24 hr after addition of poly(I)·poly(C); however, no change in CPE was detectable beyond 6 hr, so all final determinations subsequently were made at 6 hr after addition of the double-stranded RNA. The end-point dilution of interferon was the last dilution that altered cells sufficiently that 10 μg of poly(I)·poly(C) caused clearly detectable CPE, characterized by floating cells and debris. CPE was graded as follows to allow approximations of end points: −, monolayers indistinguishable from poly(I)·poly(C)-treated normal cells; ±, clearly detectable difference from control poly(I)·poly(C)-treated cells in amount of floating cells and debris, but no detectable destruction of cells in the monolayer itself; +, detectable destruction in monolayer but less than 25% of cells destroyed; ++, 25 to 75% of cells destroyed; +++, 75 to 95% of cells destroyed; ++++, monolayer completely destroyed.

As shown in Table 1, mouse serum interferon and L cell interferons with various specific activities all enhanced the susceptibility of L-929 cells to the toxicity of poly(I)·poly(C), and in each case the ratio of toxicity-enhancing activity to antiviral activity of the preparations was similar. The titers obtained by toxicity-enhancement assays were consistently two to three times those obtained by plaque-reduction assays. Although the method of reading CPE is subjective,

TABLE 1. *Comparisons of titers obtained in simultaneous assays of various mouse interferons by plaque reduction and toxicity enhancement*[a]

Interferon prepn	Expt no.	$\log_{10}$ interferon titer[b]		$\log_{10}$ ratio toxicity enhancement/ PDD_{50}-VSV
		PDD_{50}-VSV	Toxicity enhancement	
Serum	1	3.2	3.6	0.4
	2	3.3	3.7	0.4
Crude L cell (specific activity ~10^4)	1	3.5	3.8	0.3
	2	3.5	4.0	0.5
	3	3.4	3.8	0.4
	4	3.5	3.9	0.4
	5	3.7	4.0	0.3
Partially purified L cell (specific activity ~ 10^5)	1	5.1	5.5	0.4
	2	5.0	5.5	0.5
Purified L cell (specific activity ~ 5×10^6)	1	4.6	5.0	0.4
	2	4.7	5.0	0.3

[a] Monolayer cultures of L-929 cells were incubated overnight with 2 ml of serial 0.5 $\log_{10}$ dilutions of indicated interferon preparations, four cultures/dilution. Media were then removed and half of each experiment was assayed by the plaque-reduction method (see Materials and Methods); 1 ml of serum-free MEM containing 10 μg of poly(I)·poly(C) was added to each of the remaining cultures, which were then incubated at 37 C for 6 hr at which time CPE readings were made and toxicity-enhancement end points were determined.

[b] Interferon plaque-reduction titers are expressed as $\log_{10}$ of the reciprocal of the dilution depressing the number of plaques of VSV by 50% (PDD_{50}-VSV); toxicity-enhancement titers are expressed as $\log_{10}$ of the reciprocal of the last dilution showing CPE 6 hr after addition of poly(I)·poly(C).

in practice the end points are distinct, and confidence can be gained by incorporating into each series of assays an internal standard interferon preparation to which a mean titer has been obtained by repeated determinations.

Relative sensitivities of two lines of L cells to the antiviral and toxicity-enhancing activities of interferon. The previous experiments showed that the antiviral and toxicity-enhancing factors in interferon preparations, if not the same factor, at least separate together through approximately 500-fold purification. Since we recently reported that L-929 cells bind about three times more interferon than do Lpa cells and that the former are about three times more sensitive than the latter to the antiviral activity of interferon (27), it seemed possible to add supportive evidence to the interpretation that the antiviral resistance-inducing factor would indeed be the same as the toxicity-enhancing factor if the ratio of these two functions were the same in Lpa cells and L-929 cells. Experiments were therefore performed to determine whether a correlation existed between the antiviral activity and toxicity-enhancing activity of interferons in these two cell lines. Lpa cell and L-929 cell cultures were treated simultaneously with interferons. Half of the cultures were then exposed to 10 μg of poly(I)·poly(C) as previously described, and the other half were inoculated with VSV and overlaid. As shown in Table 2, L-929 cells were approximately three times more sensitive than Lpa cells to the antiviral activity of crude and purified interferon, and were also about three times more sensitive to the toxicity-enhancing activity of these preparations.

Effect of the concentration of poly(I)·poly(C) on sensitivity of toxicity-enhancement assays. We have reported that the amount of CPE induced in cells treated with a given concentration of interferon increased as the level of poly(I)·poly(C) increased (26). This suggested that by employing higher concentrations of poly(I)·poly(C) we could detect alteration of cells that had been treated with even lower concentrations of interferon than were detectable by use of 10 μg of poly(I)·poly(C), thereby increasing the sensitivity of the assay. We therefore treated L-929 cell cultures with interferons and then exposed these cultures to various concentrations of poly(I)·poly(C) or to VSV. As shown in Fig. 1, each fivefold increase in poly(I)·poly(C) to which cells were exposed gave approximately a fivefold increase in the sensitivity of the detection of cellular alteration that had been brought about by treatment with interferon. Crude interferon with a PDD_{50}-VSV titer of approximately $10^{3.5}$ assayed $10^{2.2}$, $10^{3.0}$, $10^{3.8}$, and $10^{4.4}$ when cells were "challenged" with 0.4, 2, 10, and 50 μg of poly(I)·poly(C), respectively, and purified interferon with a PDD_{50}-VSV titer of approximately $10^{4.7}$ assayed $10^{3.5}$, $10^{4.3}$, $10^{5.0}$, and $10^{5.6}$ when cells were "challenged" with 0.4, 2, 10, and 50 μg of poly(I)·poly(C), respectively. Cultures exposed to less than 0.4 μg of poly(I)·poly(C) failed to develop detectable CPE, even if they had been incubated with 10,000 PDD_{50}-VSV units of interferon. The CPE induced by 0.4 μg of poly(I)·poly(C) was slight (±), even in cells that had been treated with interferon concentrations well above the amount giving end-point toxicity enhancement. Similarly, the maximal CPE developing in cultures exposed to 2, 10, or 50 μg of poly(I)·poly(C) did not increase above +, ++, or +++, respectively, regardless of how much additional interferon was added. In each case, however, the end points were clearly delineated

TABLE 2. *Relative sensitivities of L-929 cells and Lpa cells to the antiviral and toxicity-enhancing activities of mouse interferon preparations*[a]

Interferon prepn	Log_{10} antiviral titers[b]		Log_{10} antiviral ratio (L-929/Lpa)	Log_{10} toxicity-enhancement titers[c]		Log_{10} toxicity-enhancement ratio (L-929/Lpa)
	Lpa	L-929		Lpa	L-929	
Crude L cell (specific activity $\sim 10^4$)	3.1	3.5	0.4	3.5	4.0	0.5
Purified L cell (specific activity $\sim 5 \times 10^6$)	4.2	4.7	0.5	4.5	5.0	0.5

[a] Experimental conditions were identical to those described in Table 1, except that cultures of both L-929 cells and Lpa cells simultaneously were incubated with the indicated interferon preparation. Half of the cultures of each cell type were then assayed by the plaque-reduction method for antiviral activity and half were assayed for susceptibility to toxicity of poly(I)·poly(C).

[b] Antiviral titers are expressed as log_{10} of the reciprocal of the dilution depressing the number of plaques of VSV by 50% on the indicated cells.

[c] Toxicity-enhancement titers are expressed as log_{10} of the reciprocal of the last dilution showing CPE 6 hr after addition of poly(I)·poly(C) on the indicated cells.

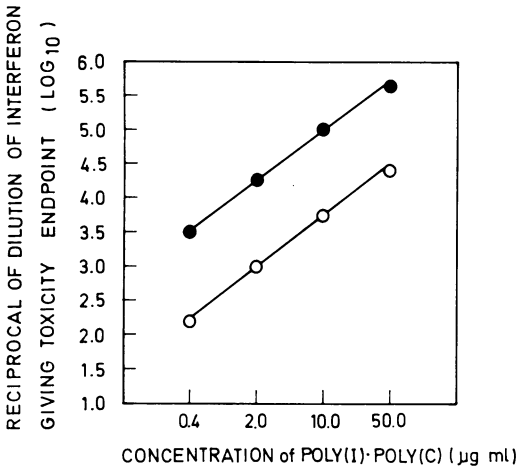

FIG. 1. *Effect of poly(I)·poly(C) concentration on the sensitivity of toxicity-enhancement assay of interferon. Monolayer cultures of L-929 cells were treated overnight with 2 ml of serial 0.5 log_{10} dilutions of either crude L cell interferon (specific activity $\sim 10^4$) (○) or purified L cell interferon (specific activity $\sim 5 \times 10^6$) (●). Duplicate cultures of each series were challenged with VSV and plaque-reduction end points were determined. Duplicate cultures of each series were also incubated for 6 hr with the indicated amount of poly(I)·poly(C) in 1 ml of serum-free MEM and toxicity-enhancement end points were determined. Plaque-reduction titers for crude and purified interferons were $10^{3.5}$ and $10^{4.7}$, respectively.*

and easily determined. Cultures exposed to 250 μg of poly(I)·poly(C) were completely destroyed (++++) if they had been incubated with interferon, but end points were not obtained with this amount of poly(I)·poly(C), because control cultures not treated with interferon developed detectable CPE after exposure to this level of double-stranded RNA.

DISCUSSION

Evidence continues to accumulate which suggests that interferons alter cells in ways other than making them resistant to viruses. Numerous reports have shown that interferon inhibits the growth of cells (9–11, 15, 17, 20, 22, 23), that it can alter their ability to respond to nonviral interferon inducers, either increasing (26, 28, 30) or decreasing (29, 36) interferon production, and that it can affect specialized functions of cells, such as phagocytosis (13), immune lysis (16), and response to mitogen (18). Additionally, interferon has been shown to alter cells in such a way that they become susceptible to destruction by double-stranded RNA molecules (26; Stewart et al., *in preparation*).

Whether these changes are separate manifestations of a single alteration brought about in cells by interferon or are separate alterations remains to be determined, but the evidence clearly presents us with the alternative interpretations that either antiviral activity is only one of the alterations brought about in cells by interferon, or antiviral activity is only one way of measuring the alteration brought about in cells by interferon.

These studies demonstrate that the alteration brought about in cells exposed to interferon which causes them to become susceptible to the toxicity of double-stranded RNA molecules can be used to assay interferons. The sensitivity of the assay can be increased by increasing the level of poly(I)·poly(C) used as "challenge," but, because 10 μg gives satisfactory sensitivity (two to three times that of the PDD_{50}-VSV assay method), economical considerations favor the use of this dose. Since the results can be obtained 6 hr after addition of the double-stranded RNA "challenge," the entire assay, from addition of interferons to reading end points, can be performed with minimal effort in less than 24 hr. Since enhanced susceptibility to double-stranded RNA has also been observed in cells of other species that had been treated with homologous interferons (26; Stewart et al., *in preparation*), this type of assay should be suitable for quantitation of other species of interferons. The speed and ease of toxicity-enhancement assays should greatly facilitate works involving screening of large numbers of samples for interferon.

We should qualify our statement that the toxicity-enhancement assay with 10 μg of poly(I)·poly(C) is more sensitive than the VSV plaque-reduction assay. Obviously, comparing clearly detectable CPE (±) to 50% plaque reduction seems inequitable. To obtain more comparable sensitivities, either (i) VSV plaque-reduction titers should be calculated from the highest dilution producing *any* reduction in plaque counts, or (ii) CPE end points should be based on the dilution of interferon which leads to 50% reduction of cells after addition of poly(I)·poly(C). However, since the CPE increases sharply below the dilution at which end-point (±) readings are obtained, reading the end point as dilutions showing 50% CPE increases the variability and subjectivity of the assay. Also, reading plaque-reduction titers as the highest dilution producing only small percentage reductions in plaque counts decreases the reproducibility of the plaque-reduction assays.

We have previously reported that L-929 cells bind more interferon than do Lpa cells and that the former are more sensitive than Lpa cells to the antiviral activity of interferon (27). Here, we show that L-929 cells are, likewise, more sensitive

than Lpa cells to the toxicity-enhancing activity of interferon. These findings suggest that the amount of binding of interferon to cells determines the amount of each of these cellular alterations, if they are indeed separate alterations rather than different manifestations of a common alteration. Previous studies have shown that interferon-induced antiviral activity requires cellular RNA and protein synthesis and takes several hours to develop (33), whereas binding of interferon to cells (27) and "priming" of cells by interferon (28) both occur rapidly, even in cells in which protein synthesis is inhibited. Further studies should show what metabolic functions are required for interferon-treated cells to become altered to the extent that they are susceptible to lysis by double-stranded RNA molecules. Preliminary experiments suggested that prolonged incubation with interferon was required for this alteration to become manifest; consequently, in these experiments cells were incubated overnight with interferon prior to addition of poly(I)·poly(C). By employing the methods described for recovering interferon from cells (27) and by determining its position on or in cells at intervals after its addition to cells, we hope to be able to evaluate the effect of its location on each alteration manifested.

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