INDUCERS OF INTERFERON AND HOST RESISTANCE, V. IN VITRO STUDIES

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Previous reports[1-6] from this laboratory recorded that double-stranded ribonucleic acid molecules from numerous sources are efficient inducers of interferon and of resistance to viral infection *in vivo* and *in vitro*. The present report describes the induction of resistance against several viruses in a variety of cell cultures by the complex of polyriboinosinic and polyribocytidylic acids (rI:rC) and presents evidence to associate such resistance with interferon induction. The inactivity of the individual homopolymers, rI and rC, is reaffirmed, and interferon induction by rI:rC *in vitro* is shown to be inhibited by exposure of cells to actinomycin D.

Materials and Methods.—(1) *Polyriboinosinic acid* (rI) and *polyribocytidylic acid* (rC) were purchased from Miles Laboratories, Elkhart, Indiana. The rI:rC complex was prepared by mixing rI and rC in equimolar concentration in phosphate-buffered saline (0.006 *M* sodium phosphate 0.15 *M* NaCl pH 7.0). (2) *Cell cultures:* The various primary, cell strain, and line cell cultures listed in Table 1 were prepared and maintained by ordinary procedures. The RK13 culture is a stable line of rabbit kidney cells, and the WI-38 and HFL cultures are diploid cell strains of human embryonic lung. The RK13 and WI-38 cultures are well documented and the HFL cell strain was developed by Dr. C. Baugh in these laboratories. Rabbit spleen cell suspensions were prepared according to Field *et al.*[2] (3) The *viruses* used were prepared from the seed stocks of this laboratory. The rhinovirus serotypes were designated according to the number system of Hamparian *et al.*[7] (4) Induction of *resistance* to viral infection was measured after overnight treatment of cell cultures with rI:rC. Following removal of inducer, interference with virus replication was measured by the plaque-reduction assay.[1] (5) *Interferon* induction in primary rabbit kidney cells or in rabbit spleen cell suspensions by rI:rC was assayed by transfer of serial dilutions of the cell supernatant fluids to RK13 cultures in Falcon flasks. This was followed by overnight incubation prior to removal and challenge with vesicular stomatitis virus (VSV) with observation for reduction in plaque formation. RK13 cells were used for assay since they were relatively insensitive to induction of resistance to VSV by rI:rC, requiring more than 1 μg/ml. Hence, these cells were unaffected by the small residual amount of rI:rC in the interferon samples. The interferon titer was the highest dilution of sample which caused at least 50% reduction in plaque formation. (6) Other pertinent methods are described in the text.

Results.—(1) *Induction of resistance in vitro to viral infection by rI:rC:* (a) *Antiviral activity in cell cultures:* Cell cultures varied in their sensitivity to rI:rC with respect to species of origin and as to whether they were primary, cell strain, or line cells. As shown in Table 1, many primary cell cultures were sensitive to rI:rC, although mouse embryo cells showed variable response. The HFL diploid human cell strain was sensitive, while the WI-38 strain of the same kind of cells was not. The RK13 culture of stable line cells showed variable sensitivity. The cause of such variability and differences is not known.

(b) *Tests of antiviral activity of rI, rC, and rI:rC preparations:* As shown in Table 2, rI:rC complex obtained from six different sources were all highly active,

TABLE 1. *Minimum amount of rI:rC required to induce resistance against VSV in various cell cultures.*

Cell culture	rI:rC (μg/ml)
Primary cell cultures	
Sensitive to rI:rC	
Rabbit kidney	0.001
Human embryonic kidney	0.04
Human amnionic membrane	0.04
Dog kidney	0.16
Calf kidney	5.25
Hamster kidney	6.5
Variable sensitivity to rI:rC	
Mouse embryo	1.3–105
Cell strains or lines	
Sensitive to rI:rC	
HFL (diploid, human fetal lung, strain)	0.6
Variable or insensitive to rI:rC	
WI-38 (diploid, human fetal lung, strain)	>105
RK13 (rabbit kidney, line)	3–105

whereas the individual homopolymers were not. The inactivity of the individual homopolymers has been confirmed by others.[8, 9] Baron *et al.*[10] reported induction *in vivo* and *in vitro* of interferon and resistance to viral infection by certain lots of "single-stranded" rI and rC obtained from P-L Biochemicals but not those obtained from Miles Laboratories. Relatively large amounts of the homopolymers were needed. Our experiences[11] with a sample of rI purchased from P-L Biochemicals confirmed the observations of Baron *et al.*[10] However, we have obtained experimental evidence that this sample of rI was contaminated with a material that appeared to be double-stranded nucleic acid. The evidences were

TABLE 2. *Tests for induction of resistance to infection with VSV in primary rabbit kidney cell culture.*

Polynucleotide	Source	Minimum dose (μg/ml) required to induce resistance
rI	Miles Laboratories, Inc.	>110
	Mann Research Laboratories	"
	Schwarz BioResearch, Inc.	"
	Sigma Chemical Co.	"
	New York University (Dr. A. J. Wahba)	"
	Merck (Research)*	"
rC	Miles Laboratories, Inc.	>100
	Mann Research Laboratories	"
	Schwarz BioResearch, Inc.	"
	Sigma Chemical Co.	"
	New York University (Dr. A. J. Wahba)	"
	Merck (Research)*	"
rI:rC	Miles Laboratories, Inc.	0.002
	Mann Research Laboratories	0.02
	Schwarz BioResearch, Inc.	0.004
	Sigma Chemical Co.	0.008
	New York University (Dr. A. J. Wahba)	0.002
	Merck (Research)*	0.002

* Prepared by Merck Process Research.

several: (*i*) The "activity" of the single homopolymers was confined to one source. (*ii*) One lot of "active" rI available to us from P-L Biochemicals was shown to contain a double-stranded contaminant, based on the fact that the "activity" was destroyed by treatment with pancreatic RNase (10 μg/ml) at 35°C for 24 hours (rI was not degraded by this treatment) and that the "activity" was destroyed by heating at 65°C with 1.5 per cent formaldehyde (rI is inert to reaction with formaldehyde and could still form inducer when complexed with rC). Formaldehyde does cause irreversible denaturation of complexed RNA by binding with amino groups of certain nucleic acid bases. (*iii*) Synthetic rI and rC prepared in these laboratories using the relatively crude P-L polynucleotide phosphorylase were active, whereas the homopolymers prepared using purified enzyme were not active when tested singly. This would suggest that the "activity" was contributed by the P-L enzyme used to prepare the homopolymers.

(c) *Activity against several viruses:* Table 3 shows that rI:rC was active in microgram or millimicrogram amounts in inducing resistance to eight different viruses tested in human embryonic or rabbit kidney cell cultures. Such activity was not due to direct antiviral effect of rI:rC on virus. Thus, VSV at 10^6 PFU/ml was incubated 18 hours at 35°C in 0.3 per cent bovine serum albumin alone or containing 2.6 μg rI:rC/ml. No difference in infectivity titer betweeen the rI:rC treated and untreated VSV was noted upon titration on primary rabbit kidney, mouse embryo, and RK13 cell cultures.

(d) *Retention of resistance following removal of rI:rC:* Primary rabbit kidney cells were treated overnight at 35°C in the presence of serial twofold dilutions of rI:rC. Half the cultures were decanted and half were washed free of rI:rC prior to challenge with VSV. The activity titer of the rI:rC was 0.006 μg/ml regardless of the retention or removal of residual inducer just prior to virus challenge.

(e) *Kinetics of induction of resistance to viral infection:* Cell cultures of primary rabbit kidney were treated with 0.1 μg rI:rC per ml for variable time periods after which residual inducer was removed by washing. Half the cultures representing each time period were challenged with VSV immediately following

TABLE 3. *Minimum amount of rI:rC required to induce resistance against various viruses in primary human and rabbit cell cultures.*

Virus	rI:rC (μg/ml)* Human embryonic kidney	Rabbit kidney
Vesicular stomatitis virus	0.04	0.001
Vaccinia	—	0.001
Herpes simplex	—	0.1
Rhinovirus 1 (strain JH)	5.6	—
11 (strain 68)	0.8	—
20 (strain 225)	3.0	—
42 (strain 457)	0.8	—
47 (strain 425)	0.8	—

* Studies with vesicular stomatitis virus, vaccinia, and herpes simplex were carried out by plaque reduction. For tests for resistance to rhinoviruses, tube cultures of primary human embryonic kidney cells were incubated 18 hr at 33°C. After removal of medium, cultures were challenged with about 10 $TCID_{50}$ of rhinovirus, incubated at 33°C, and observed for inhibition of development of cytopathology.

TABLE 4. *Kinetics of development of resistance to VSV in primary rabbit kidney cell cultures treated with rI:rC.*

Incubation time with rI:rC (0.1 μg/ml) (hr)	Av. Plaque-Forming Units/Culture after Three Days of Incubation — Time of Challenge with VSV: Immediately after washing*	18 hr after washing
1	52†	0
2	31	1
3	0	0
4	0	0
5	1	0
18	0	—
Control (no rI:rC)	28	—

* The last washing was routinely tested for and found free of residual rI:rC.
† Greater plaque formation compared with untreated controls was also noted when untreated cultures were washed just prior to challenge.

washing, and the remainder were challenged after further overnight incubation in medium that was free of rI:rC. In Table 4 it is seen that a minimal exposure time of three hours was needed for development of complete resistance. On the other hand, such resistance was obtained after a single hour's exposure to rI:rC, provided that the cultures were allowed to incubate for an additional 18 hours in the absence of the inducer. This showed that exposure of the cells to rI:rC alone did not suffice to induce resistance. Instead, exposure followed by more than an additional hour of incubation time was needed whether or not rI:rC remained.

(2) *Induction of interferon in vitro by rI:rC:* (a) *Induction and characterization of interferon induced in rabbit spleen cells:* Rabbit spleen cells in suspension were treated with rI:rC at a final concentration of 10.5 μg/ml. Individual rI and rC homopolymers tested separately at equivalent concentrations were inactive, whereas the fluid from cell suspensions treated for 7.5 hours with rI:rC showed a titer of viral inhibitory substance of 1:128, as measured in the VSV plaque assay on RK13 cells. The inhibitory substance was characterized biologically and physically by procedures described earlier.[1-4] The activity was demonstrable in homologous rabbit RK13 cells (titer 1:128) but not in primary mouse or chick embryo cells in culture (titer < 1:8). Treatment of the inhibitor, which had been partially purified by chromatography on CM-Sephadex, with 50 μg crystalline trypsin per ml at 35°C for four hours reduced the inhibitory titer from 1:128 to < 1:2. The isoelectric point of the purified inhibitor was 6.9–7.0 based on the CM-Sephadex elution profile,[12] and the molecular weight was 43,000 (± 10%) as measured by chromatography on Sephadex G-100. These findings sufficed to identify the active substance as interferon.

(b) *Induction and characterization of interferon produced in primary rabbit kidney cells:* Primary cell cultures of rabbit kidney were treated with rI:rC at a concentration of 1.05 μg/ml. Fluids were withdrawn from the cell cultures at increasing time periods and assayed for viral inhibitory substance in the VSV plaque assay on RK13 cells. The findings shown in Figure 1 were plotted in terms of per cent reduction in plaque formation by the samples taken after one to seven hours compared with the control sample collected immediately following

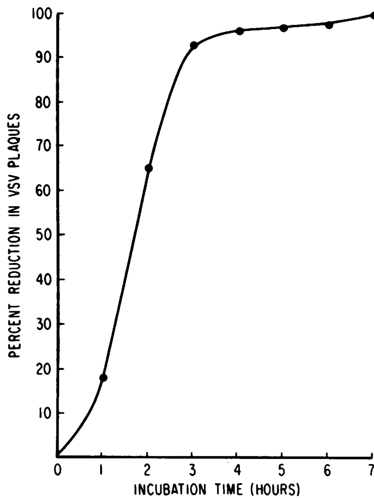

FIG. 1.—Induction of interferon in primary rabbit kidney cell cultures by treatment with rI:rC (1.05 μg/ml).

exposure to rI:rC. Antiviral activity was demonstrable as early as one hour after treatment with rI:rC and by three hours there was at least 93 per cent suppression of plaques. As with direct induction of resistance described above, rI or rC separately did not induce antiviral activity in the fluids. The antiviral substance in the fluids from the rI:rC treated cultures was active on RK13 cells (titer 1:4), but showed no plaque-reducing capacity on the heterologous mouse embryo, chick embryo, or monkey kidney cells. This antiviral activity seen on the homologous RK13 cells was completely destroyed by trypsin treatment.

(3) *Inhibition of interferon production by actinomycin D:* (a) *Rabbit spleen cell suspensions:* Rabbit spleen cells in suspension were treated for one hour at 35°C with actinomycin D at a concentration of 5 μg/ml. The cells were then incubated for seven hours at 35°C with 10.5 μg rI:rC per ml following removal of residual actinomycin D by repeated washing. Appropriate controls were included in which the cells were similarly treated except for the exclusion of either actinomycin D or rI:rC. With VSV and RK13 cells, the interferon content of the culture fluids was determined by plaque assay. Table 5 shows that pretreatment with actinomycin D totally inhibited subsequent production of interferon upon exposure of the cells to rI:rC. This suppression was not due to inhibition, by residual actinomycin D, of the activity of interferon in inducing resistance to virus, since it was found in additional tests that there was insufficient actinomycin D left after washing the treated cells to block the antiviral action of added interferon.

(b) *Primary rabbit kidney cell cultures:* Primary rabbit kidney cell cultures were incubated for two hours at 35°C with fresh medium containing 0.5 μg actinomycin D per ml. The actinomycin D was then removed by repeated washing, and medium containing 1.05 μg rI:rC per ml was added. The fluids

TABLE 5. *Inhibition of rI:rC induced in vitro interferon production by pretreatment of cells with actinomycin D.*

Cell culture system	Treatment		Bioassay	
	Actinomycin D	rI:rC	Av. plaque count	Dilution titer
Rabbit spleen cell suspensions	None	10.5 μg/ml, 7 hr	—	1:128
	5 μg/ml, 1 hr	10.5 μg/ml, 7 hr	—	<1:8
	5 μg/ml, 1 hr	None	—	<1:8
Primary rabbit kidney cell cultures	None	1.05 μg/ml, 5 hr	1	—
	0.5 μg/ml, 2 hr	1.05 μg/ml, 5 hr	47	—
	0.5 μg/ml, 2 hr	None	42	—
	None	None	67	—

were removed from the culture after incubation at 35°C for five hours and were tested for presence of interferon in the VSV plaque system. Table 5 shows that the actinomycin D completely suppressed the production of interferon by rI:rC added subsequently. Rigorous removal of residual actinomycin D from the cultures prior to addition of rI:rC was carried out in order to eliminate the possibility for actinomycin D interference with the antiviral activity of interferon in the assays for interferon.

Discussion.—It is evident that rI:rC is capable of inducing resistance to viral infection in a wide variety of cells in culture, but there may be considerable difference in the amount of inducing substance required to accomplish this. Primary cell cultures of human embryo, human amnion, rabbit kidney, and dog kidney were most sensitive to induction, while similar cultures of calf and hamster kidney required far greater amounts of rI:rC. Variable sensitivity to induction was shown for primary mouse embryo cells in culture and for the RK13 stable line of rabbit kidney cells. It was of some interest that the HFL cell strain of diploid human embryonic lung cells was highly sensitive to induction by rI:rC, whereas the WI-38 cell strain of similar origin was not. The requirement of double-strandedness for induction of resistance and interferon was reaffirmed. Evidence was presented suggesting that induction by single-stranded polynucleotides[10] was due to apparent contamination with a double-stranded material.

Present evidence in the studies with rI:rC indicates that the *in vitro* induction of resistance to viral infection by the polymer is dependent upon new synthetic processes and is associated with the induction of interferon. It is presumed that such resistance to virus may be due to the production of interferon, but this relationship is not proved. The evidence, however, is consistent with this thesis. Thus, interferon and resistance to viral infection are both induced by rI:rC. Resistance to viral infection, like that resulting from addition of interferon, is broad-spectrum with respect to viral species. Development of resistance to virus, like the production of interferon, requires an interaction between the rI:rC and the cell, and a time period is required both for the appearance of interferon and for the development of resistance. Additionally, the polynucleotide has no direct destructive effect upon the virus and there is no simple interference with infection of the cell by virus such as the masking or destruction of receptors on the cell.

Interferon induction in rabbit spleen cell suspensions and in primary rabbit kidney cell cultures was inhibited by pretreatment of the cells with actinomycin D, indicating apparent requirement for DNA-dependent RNA synthesis.[13] Whether rI:rC induced *de novo* biosynthesis of interferon *in vitro* cannot as yet be answered. However, the findings suggest that rI:rC induction of interferon may well be comparable to induction of protein synthesis as suggested by Jacob and Monod.[14]

Recently, Youngner and Hallum[15] reported that the induction of interferon by rI:rC in mice was not prevented by prior treatment with cycloheximide, whereas that induced by Newcastle disease virus (NDV) was so inhibited. They concluded, therefore, that rI:rC served only to release preformed interferon in the intact animal. Such data are in disagreement with the present findings and

with the previous reports by Vilček *et al.*[8] and by Falcoff and Bergoff[9] who showed that induction of interferon by rI:rC in primary rabbit kidney and human leukocytes in cell culture is inhibited by actinomycin D. Additionally, Smith and Wagner[16] showed that actinomycin D and puromycin prevented induction or synthesis of interferon in rabbit macrophages stimulated with endotoxin or NDV. It should be noted that the findings in intact rabbits and mice are difficult to interpret since the inhibitory drugs which have been used may exhibit undefined activities in the various kinds of cells in the metazoan host. For example, the cycloheximide blockage of interferon induction by NDV, as reported by Youngner and Hallum,[15] may be due to the prevention of synthesis of protein essential to the formation of the double-stranded RNA rather than in the inhibition of interferon induction by double-stranded RNA. The more definitive approach to answering the questions relating to mechanism for interferon induction would appear to derive from studies in cell culture systems *in vitro*.

Summary.—Complexed polyriboinosinic (rI) and polyribocytidylic (rC) acids are highly active in inducing resistance to viral infection in cell culture. Such resistance was broad-spectrum with respect to kinds of viruses, but the amount of rI:rC required for induction varied considerably for different cells and was not always consistent for a particular kind of cell. Single-stranded rI and rC were inactive *in vitro* and evidence was presented to indicate that the purported activity of such materials was due to presence of a contaminant that was double-stranded. Development of resistance to viral infection following rI:rC treatment was accompanied by production of interferon. Induction of interferon by rI:rC *in vitro* was prevented by prior treatment of cells with actinomycin D, indicating an apparent requirement for DNA-dependent RNA synthesis.

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[1] Lampson, G. P., A. A. Tytell, A. K. Field, M. M. Nemes, and M. R. Hilleman, these PROCEEDINGS, **58**, 782 (1967).

[2] Field, A. K., A. A. Tytell, G. P. Lampson, and M. R. Hilleman, these PROCEEDINGS, **58**, 1004 (1967).

[3] Tytell, A. A., G. P. Lampson, A. K. Field, and M. R. Hilleman, these PROCEEDINGS, **58**, 1719 (1967).

[4] Field, A. K., G. P. Lampson, A. A. Tytell, M. M. Nemes, and M. R. Hilleman, these PROCEEDINGS, **58**, 2102 (1967).

[5] Field, A. K., A. A. Tytell, G. P. Lampson, and M. R. Hilleman, *Bacteriol. Proc.* (1968), 68th Annual Meeting, American Society for Microbiology, Detroit, 1968, Abstr. V6.

[6] Hilleman, M. R., *J. Cellular Physiol.*, **71**, 43 (1968).

[7] Hamparian, V. V., M. B. Leagus, and M. R. Hilleman, *Proc. Soc. Exptl. Biol. Med.*, **116**, 976 (1964).

[8] Vilček, J., M. H. Ng, A. E. Friedman-Kien, and T. Krawciw, *J. Virol.*, **2**, 648 (1968).

[9] Falcoff, E., and R. P. Bergoff, personal communication, as reported in Interferon Scientific Memorandum no. 59, April 1968.

[10] Baron, S., N. N. Bogomolova, H. B. Levy, A. Billiau, and C. E. Buckler, personal communication, as reported in Interferon Scientific Memorandum no. 55, March 1968.

[11] Tytell, A. A., G. P. Lampson, A. K. Field, M. M. Nemes, and M. R. Hilleman, personal communication, as reported in Interferon Scientific Memorandum. no. 81, May 1968.

[12] Lampson, G. P., and A. A. Tytell, *Anal. Biochem.*, **11**, 374 (1965).

[13] Reich, E., R. M. Franklin, A. J. Shatkin, and E. L. Tatum, *Science*, **134**, 556 (1961).

[14] Jacob, F., and J. Monod, *J. Mol. Biol.*, **3**, 318 (1961).

[15] Youngner, J. S., and J. V. Hallum, *Virology*, **35**, 177 (1968).

[16] Smith, T. J., and R. R. Wagner, *J. Exptl. Med.*, **125**, 559 (1967).