Effect of Poly I:C on Transformation by Rous Sarcoma Virus[1] (36373)

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Early *in vivo* studies utilizing chicks or chick embryo chorioallantoic membrane indicated that Rous sarcoma virus (RSV) tumorigenesis was inhibited by interferon (1). In addition, interferon preparations induced by RSV (2) or Chickungunya virus (3) were shown to significantly reduce RSV focus formation in tissue culture. With regard to other RNA tumor viruses, it has been shown that murine leukemia is blocked by interferon (4) as well as by viral (5) and nonviral interferon inducers (6, 7). Furthermore, transformation *in vitro* (8–11) and *in vivo* (12) by murine sarcoma virus is blocked by interferon and interferon inducers.

This paper describes the inhibitory effect of polyinosinic:polycytidylic acid (poly I:C) on focus formation and virus production in chick embryo fibroblasts infected with RSV. Our data indicate that early events involved in the establishment of transformation are blocked by this drug.

Materials and Methods. Cells and Virus. Methods used in the handling of chick embryo fibroblast cultures, and in the assay of RSV by focus formation have recently been summarized (13). All cultures were prepared from Kimber embryos which had been shown to be free of Rous interfering factor (14), and susceptible to all subgroups of avian RNA tumor viruses. A cloned preparation of the helper independent (15) Bratislava-77 virus (16), RSV(B77), was used in most experiments. No differences were found among RSV(RAV-1), RSV(RAV-2) and RSV(B77) with regard to transforming efficiency on poly I:C-treated cultures. Plaque assays of vaccininia virus on confluent, secondary chick fibroblast cultures were performed according to established procedures (17).

Poly I:C assays. To measure the effect of poly I:C and chick interferon, secondary cultures were initiated at 1×10^6 cells per 60-mm plastic Petri dish. After 48–72 hr, when cultures were confluent, growth medium (Medium 199 supplemented with 5% calf serum and 0.1% beef embryo extract) was removed and replaced with 3 ml maintenance medium (Medium 199 supplemented with 1% calf serum) containing poly I:C plus DEAE dextran (DEAE dx) or chick interferon. DEAE dx strongly enhances the production of interferon and viral resistance which is induced by polynucleotides (18). After a further 24 hr, cultures were trypsinized, reseeded in growth medium at 1×10^6 cells per dish and infected within 2 hr. Dishes were overlayed with agar medium within 18 hr and examined for foci at 5–6 days postinfection.

Growth-rate measurements. To obtain data on growth rate, cultures were treated as usual with poly I:C. After 24 hr, cells were removed with trypsin, reseeded at 5×10^5 cells per 35-mm plastic dish and left under liquid medium. At time intervals thereafter, cells from duplicate dishes were removed by trypsinization and counted immediately in 1% trypan blue.

Polymers. Poly I:C was obtained in solution from Microbiological Associates. DEAE dx (Pharmacia) was prepared in water. Poly I and poly C (P. L. Biochemicals) were prepared in phosphate buffered saline (0.01 *M* sodium phosphate, 0.15 *M* sodium chloride, 0.005 *M* magnesium chloride, pH 7.0)

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TABLE I. Effect of Poly I:C Concentration on Transformation by RSV.[a]

Poly I:C (μg/ml)	DEAE dx (μg/ml)	Foci/dish[b]	% Reduction
1.0	10.0	195	83
1.0	1.0	70	94
0.1	1.0	310	73
0.01	1.0	940	19
1.0	0.1	1090	6
0.0	0.0	1155	—

[a] Growth medium was removed from confluent, secondary cultures and replaced by maintenance medium containing poly I:C and DEAE dx at the concentrations shown. After 24 hr, cultures were transferred and infected with RSV.

[b] Average of duplicate dishes.

and poly dG:dC (Miles) was dissolved in 0.01 *M* sodium citrate, 0.1 *M* sodium chloride. Polymers were always added to cultures at 1% of the total culture volume.

Interferon. Chickungunya virus-induced and Sindbis virus-induced chick interferons were kindly supplied by G. E. Gifford and P. I. Marcus, respectively. Chickungunya virus-induced interferon had an activity of 20 units per ml against vaccinia virus, and Sindbis virus-induced interferon had an activity of 1–2000 units per ml against vesicular stomatitus virus.

Results. Effect of poly I:C on RSV transformation. In initial experiments, poly I:C was added to growing secondary cultures 24 hr after cell seeding. The cultures were transferred and infected 24 hr later. RSV transformation as well as virus production were both reduced by only 70% with poly I:C at 1 or 10 μg/ml plus DEAE dx at 1 μg/ml. Also, Chickungunya virus-induced interferon at approximately 30 units per ml was ineffective. Two changes were made simultaneously in the protocol which significantly increased inhibition to transformation by poly I:C. These changes were (a) treatment of 48–72 hr confluent cultures instead of 24 hr nonconfluent ones, and (b) treatment in the presence of maintenance medium rather than growth medium. It has been reported that medium containing low concentrations of serum likewise increases the efficiency of interferon by 100-fold and that cell aging increases the efficiency by 10-fold (19, 20). Others, however, have shown that interferon efficiency is not increased in aged cultures (21). It is not known from our data whether aging, or confluency *per se*, or low serum is responsible for the increased efficiency of poly I:C.

Under the conditions of low serum and confluency, it is seen that poly I:C at 0.1 μg/ml is sufficient to reduce RSV transformation by 70% (Table I). Doses of 1 and 10 μg/ml further reduced the transforming efficiency by 90 and 97–98%, respectively

TABLE II. Effect of Time of Addition of Poly I:C on Transformation by RSV.[a]

Time	Foci/dish[b]	% Reduction
−24 hr	67	97
0 hr	1980	17
+17 hr	2105	12
untreated	2400	—

[a] Growth medium was removed from confluent, secondary cultures and replaced by maintenance medium containing poly I:C and DEAE dx, both at 10 μg/ml. Parallel cultures received neither poly I:C nor DEAE dx. After 24 hr, cultures were transferred and infected with RSV. Either at the time of infection (0 hr) or 17 hr postinfection, poly I:C and DEAE dx were added to cultures not yet exposed to these additives.

[b] Average of duplicate dishes.

TABLE III. Duration of Poly I:C Exposure Necessary for Inhibition of Transformation by RSV.[a]

Exposure time	Foci/dish[b]	% Reduction
10 min	430	60
3 hr	125	88
9 hr	45	96
17 hr	25	98
23 hr	30	97
untreated	1065	—

[a] Growth medium was removed from confluent, secondary cultures and replaced by maintenance medium containing poly I:C and DEAE dx, both at 10 μg/ml. Media were removed at the times indicated, cultures were rinsed 3 times with 3 ml Tris buffer and refed with 3 ml maintenance medium. Twenty-four hours after the addition of poly I:C and DEAE dx, cultures were transferred and infected with RSV.

[b] Average of duplicate dishes.

TABLE IV. Effect of Poly I:C on Replication of RSV.[a]

Fluid dilution	Foci/dish: Untreated	Foci/dish: Treated	% Reduction
10^{-1}	TNTC[b]	560	—
10^{-2}	330	50	85

[a] Cultures were treated in the standard fashion (see Table I and Materials and Methods) with poly I:C and DEAE dx, both at 1 μg/ml. After 24 hr, cultures were transferred and infected with RSV. After 48 hr, culture fluids from duplicate dishes were harvested, diluted, and 0.1 ml was assayed for transforming activity.

[b] Too numerous to count.

(Tables I–III). For comparison, plaque formation by vaccinia virus was reduced by 100% with poly I:C at 0.6 μg/ml (plus DEAE dx at 10 μg/ml). Poly I:C was effective against RSV transformation only if given before infection (Table II). Exposure of cells to the drug at the time of infection or 17 hr later failed to significantly prevent transformation. Furthermore, only a brief exposure of cells to poly I:C was necessary to reduce transformation (Table III). A reduction of 60% was obtained with only 10 min of exposure, and maximum reduction was obtained with an exposure time between 3 and 9 hr. DEAE dx was essential for poly I:C activity in the RSV system, although it had no inhibitory properties in either the RSV or vaccinia system when used alone.

There is apparently no difference between helper dependent and helper independent strains of RSV with regard to inhibition of transformation by poly I:C, as both were inhibited by approximately 90% with poly I:C at 1 μg/ml (see Materials and Methods).

In order to determine the effect of poly I:C on RSV replication, cells were pretreated with poly I:C and infected after 24 hr. After a further 48 hr, culture fluids were harvested, diluted and assayed for transforming activity. Table IV shows that pretreatment of cells with poly I:C reduces RSV replication to the same extent as it reduces transformation, *i.e.*, 85% with poly I:C at 1 μg/ml.

Effect of interferon on RSV transformation. In marked contrast to poly I:C, interferon had no significant activity against RSV transformation (Table V). Chickungunya virus-induced interferon at approximateiy 30 units was without effect on RSV transformation although it was quite effective in reducing plaque formation by vaccinia virus. In addition, Sindbis virus-induced interferon at 300 units failed to reduce transformation.

Culture fluids collected from cells exposed for 24 hr to poly I:C at 1.0 μg/ml were essentially ineffective in reducing RSV transformation. Plaque formation by vaccinia virus, however, was reduced 95% with a 1:40 dilution of this fluid. The inhibition obtained with this fluid is probably due to interferon and not residual poly I:C, as poly I:C is rapidly absorbed by chick cells (18). Furthermore, our experience with the sensitivity of RSV transformation to culture fluids collected from poly I:C-treated cells indicates that greater than 99% of a poly I:C dose of 10 μg/ml has been removed from the culture fluid between 9 and 18 hr after polymer addition.

Cytotoxicity and growth rate. Cytotoxicity was frequently observed in poly I:C-treated cultures, especially those receiving poly I:C at 10 μg/ml. This was characterized by a rounding up of the fibroblast with subsequent detachment from the dish, and by the presence of large, giant-like cells. With poly I:C at 1 μg/ml, however, this effect was sometimes absent or very minimal and transformation was still reduced by approximately 90%. The degree of cytotoxicity observed with poly I:C at 0.1 μg/ml was usually slight, and again, transformation was still significantly reduced. To ascertain whether the effect of poly I:C was truly due to an inhibition of transformation or was simply due to cytotoxicity, confluent cultures were treated with poly I:C for 24 hr, removed by trypsinization and the number of viable cells according to trypan blue exclusion was determined. The number of viable cells recovered from cultures treated with poly I:C at 10 and 1 μg/ml were routinely reduced by approximately 60–70 and 50%, respectively, in comparison with untreated cultures. Cultures

TABLE V. Effect of Chick Interferon on Transformation by RSV and Replication of Vaccinia.[a]

		RSV		Vaccinia	
Culture fluid	Fluid dilution	Foci/dish[b]	% Reduction	Plaques/flask[c]	% Reduction
Control	1:2	1265			
Interferon	1:2	915	27		
Control	1:5			137	
Interferon	1:10			23	83
Interferon	1:20			47	66
Interferon	1:40			63	54
Interferon	1:80			80	41

[a] Growth medium was removed from confluent, secondary cultures and replaced with 3 ml maintenance medium containing culture fluid from Chickungunya virus-stimulated chick cultures (interferon fluid) or fluid from parallel, nonstimulated cultures (control fluid). After 24 hr, cultures were either transferred and infected with RSV or infected with vaccinia virus without transfer according to Lindenmann and Gifford (16).

[b] Average of duplicate dishes.

[c] Average of triplicate flasks.

treated with poly I:C at 1 μg/ml occasionally, however, did not exhibit a reduction in the number of recovered, viable cells.

If viable cells from untreated cultures and from cultures treated with poly I:C at 1 μg/ml were reseeded at the same number per dish and growth measurements were then made, it is apparent that there was no significant difference in the growth rate between treated and untreated cells (Fig. 1). To eliminate the possibility that poly I:C pretreatment followed by infection produced an additive effect which was toxic, a portion of treated and untreated cultures was infected at a multiplicity of 1.0 soon after reseeding. It is apparent that infection of poly I:C-treated cells causes no alteration in growth rate relative to untreated cells (Fig. 1).

Specificity of ribonucleotide polymer. Tests were conducted to determine whether the effect obtained with poly I:C was specific for this polymer, and not for other nucleotide polymers. Neither poly I, nor poly C, nor poly dG:dC was successful in significantly reducing transformation (Table VI). These polymers are also ineffective in reducing replication of nontumor viruses (18). Finally, acid hydrolysis with subsequent neutralization of the poly I:C preparation produced a solution which failed to have an effect on RSV transformation. This finding excludes the possibility that a factor in the preparation of low molecular weight is responsible for the effect of this preparation on RSV transformation.

Discussion. The inhibition of focus formation by poly I:C could be due either to a true inhibition of transformation or to a cytotoxic effect resulting in the inability of the transformed cell to grow and thus form a focus. The cytotoxic effect of poly I:C (22–24), as well as the growth inhibitory property of interferon (25, 26) is well-documented. The mechanism of poly I:C inhibition of RSV transformation does not seem to be due to a cytotoxic effect for the following reasons:

(a) Pretreatment of cells with poly I:C at 0.1 μg/ml consistently reduces transformation by approximately 70%, but usually produces no cytotoxicity. Moreover, poly I:C at 1 μg/ml occasionally produces little or no cytotoxicity although transformation is reduced by approximately 90%.

(b) There is no difference in the rate of growth between cultures treated with poly I:C at 1 μg/ml and untreated cultures. Moreover, infection of these pretreated cells at a multiplicity of 1.0 with RSV produces no detectable alteration in growth rate.

(c) Only 10 min of exposure of cells to poly I:C at 10 μg/ml is sufficient to signifi-

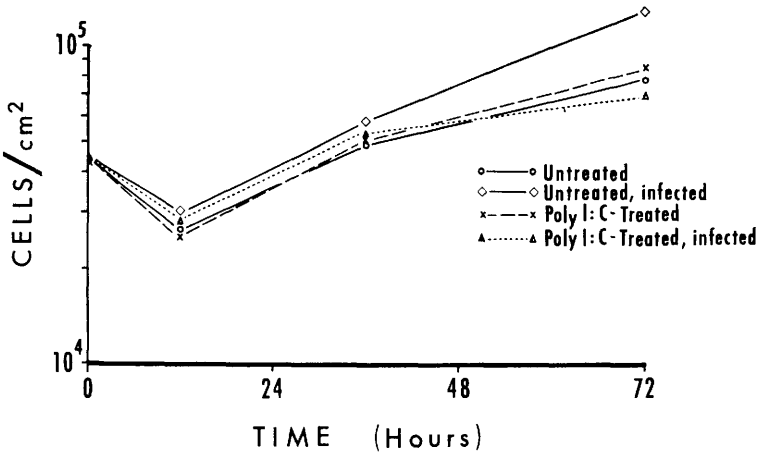

FIG. 1. Effect of poly I:C on the growth rate of normal and infected chick fibroblasts. Cultures were treated in the standard fashion with poly I:C and DEAE dx, both at 1 μg/ml (see Table I and Materials and Methods). After 24 hr, cultures were trypsinized, reseeded, infected at a multiplicity of 1.0, and duplicate dishes were taken for viability counts at the times indicated.

cantly reduce transformation and an exposure time of only 10 min produces no apparent cytotoxicity.

(d) Addition of the drug simultaneously with, or 17 hr after infection has no significant effect on transformation even when poly I:C at 10 μg/ml is used. This finding must be interpreted with caution, however, as the cells at the time of infection and at 17 hr postinfection are in an active state of growth. Cells receiving the drug 24 hr before infection, on the other hand, are contact inhibited. Data in the literature on interferon (2) as well as our own data on poly I:C indicates that the efficiency of these substances is depressed in rapidly growing cultures. Even so, preliminary experiments with poly I:C showed that transformation and virus production were reduced by approximately 70% when poly I:C at 1 μg/ml was added to cultures which were in a state of growth approximately equivalent to that of the cultures receiving poly I:C at 17 hr postinfection. In the latter case, however, drug exposure time was only 1 hr whereas in the former case it was 24 hr. It would be difficult to perform the above type of experiment since transformation by RSV is dependent on active cell division (27). Experiments are in progress, however, to determine conclusively the effect of the time of addition of poly I:C on RSV transformation.

Surprisingly, poly I:C at 10 μg/ml was not markedly toxic when added to cultures at the time of infection or at 17 hr postinfection. The decreased cytotoxicity in these cases, as compared with that obtained when the drug was added 24 hr before infection, possibly reflects the differences in the state of growth between these times.

The mechanism of poly I:C inhibition of RSV transformation may still be due to interferon although we were not able to significantly inhibit RSV transformation with exogenous interferon, nor were we able to detect an inhibitor of RSV transformation in fluids from poly I:C-treated cultures. It is known that the amount of interferon released

TABLE VI. Specificity of Nucleotide Polymer.[a]

Nucleotide polymer	Foci/dish[b]	% Reduction
none	590	—
poly I:C	130	78
poly I	470	20
poly C	730	0
poly dG:dC	579	2

[a] Cultures were treated in the standard fashion with DEAE dx plus a polymer solution, both at 1 μg/ml (see Table I and Materials and Methods). After 24 hr, cultures were transferred and infected with RSV.

[b] Average of duplicate dishes.

into culture fluids from poly I:C-treated chick cultures is quite small (18). For this reason, extracts from poly I:C-treated cultures should be tested for their ability to inhibit RSV transformation as it is possible that high intracellular levels of interferon are induced even though the amount appearing in the culture fluid is small. Also, in our assay the effectiveness of exogenous interferon was probably decreased at the time of infection as the cells at this time were in a rapid state of growth.

Experiments are in progress to further determine the mechanism of poly I:C inhibition of RSV formation, to retest the effect of poly I:C and especially interferon under different culture conditions, and to search for inhibitors of RSV transformation in extracts from poly I:C-treated cultures.

Summary. Treatment of chick embryo fibroblasts with poly I:C significantly inhibits the ability of RSV to transform and to replicate in these cells. Under identical assay conditions, however, interferon is relatively ineffective in decreasing either of these parameters. The effect of poly I:C is obtained only when the drug is given before infection, suggesting that very early events involved in the fixation of transformation are inhibited. Last, significant inhibition by poly I:C is achieved with as little an exposure time as 10 min.

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