Comparison of the Antitumor Effect of Interferon and Interferon Inducers[1] (35304)

JOHNG S. RHIM AND ROBERT J. HUEBNER

Microbiological Associates, Inc., National Institute of Allergy and Infectious Diseases, and National Cancer Institutes, National Institutes of Health, Bethesda, Maryland 20014

Broad antitumor activity against many transplantable and virus-induced tumors has recently been reported for interferon (IF) and IF inducers (1–13). However, direct comparison of the antitumor effect of IF and various IF inducers has not yet been reported.

We report the results of investigations designed to compare the antitumor effect of IF and IF inducers: NDV, statolon, polyribonucleic acids (poly I·poly C and poly A·poly U) *in vitro* and *in vivo* under defined conditions. The possible mechanism of antitumor effects were also studied.

Materials and Methods. Viruses. Concentrates of mouse tumors (14) induced by Moloney sarcoma virus (M-MSV) were used. Its infectivity titer was $10^{4.0}$ FFU/ml.

Newcastle disease virus (NDV) (Hertz strain) was propagated in 11-day-old chicken embryos and stored at −60°. Its hemagglutinin titer was 5000 units/ml and its infectivity titer was $10^{9.3}$ PFU/ml.

Virus assay. Murine sarcoma virus was assayed in secondary Swiss NIH mouse embryo tissue culture (NIH-METC). Altered cell foci (15) were counted with an inverted Zeiss microscope 5–7 days after sarcoma virus inoculation.

Cell cultures and media. Primary cultures of NIH-METC were prepared as previously described (16). Growth and maintenance medium was 10% fetal bovine serum in Eagle's minimum essential medium (EMEM) with 2 n*M* glutamine, and antibiotics (100 units of penicillin and 100 μg of streptomycin/ml).

Mouse L cells were grown in Eagle's No. 2 medium containing 10% fetal bovine serum. They were maintained in Eagle's No. 2 medium plus 2% fetal bovine serum.

Tumor cell lines. The hamster tumor cell line used in this study was derived from hamster tumors induced by Rauscher leukemia virus-transformed hamster embryo cells (RHT-1) (17).

The mouse Ehrlich ascites tumor, EAC_3H, was obtained from Dr. H. du Buy, who received the tumor from Dr. R. B. Roberts. It was passaged twice through newborn rats to eliminate the contaminating lactic dehydrogenase virus. It was subsequently passed weekly through CDF_1 mice, as a 1:5 dilution, intraperitoneally in 0.2-ml amounts. Treatment schedules are indicated individually for each experiment.

Animals. Newborn NIH Swiss mice and golden Syrian hamsters and 15–20-g CDF_1 mice were obtained from the Animal Production Section of the National Institutes of Health.

Statolon. The statolon was supplied through the kindness of W. J. Kleinschmidt, Lilly Research Laboratoy, Indianapolis. A stock solution was prepared as 1 ml of statolon/ml, diluted in 2% fetal bovine serum in EMEM with antibiotics.

Polyribonucleic acids. The single-stranded homopolymer polyriboinosinic acid (poly I) and polyribocytidylic acid (poly C) were purchased from P-L Biochemicals, Inc., Milwaukee, Wisconsin. The double-stranded poly I·poly C was prepared at a concentration of 1 mg/ml by complexing the 2 homopolymer nucleotides in equimolar concentration in 0.01 *M* phosphate buffered saline (PBS), at pH 7.2, containing 5×10^{-3} *M* MgCl. Complex formation was confirmed by determining

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the hypochromic effect (12).

Interferon. Mouse serum IF was prepared in adult NIH Swiss mice with Hertz strain of NDV (18). Its titer was $10^{4.2}$ units/ml. In this assay system, the NIH reference mouse IF titered $10^{4.5}$ units/ml.

Interferon assay. The titer of IF was determined as the reciprocal of the greatest dilution of the sample which inhibited yield of GD 7 virus hemagglutinin by 0.5 $\log_{10}$ in mouse L cells during a 1-step growth cycle (Oie *et al.*, personal communication).

Treatment with IF and IF inducers. In the *in vitro* study, secondary NIH-METC cultures, no more than 24 hr old, were pretreated for 24 hr at 37°. The cultures were then washed three times with Hanks' balanced salt solution (HBSS), supplied with fresh growth medium, and inoculated with MSV. Foci of transformed cells were counted 5–7 days later.

In the *in vivo* study, in mice and hamsters, treatment with IF or inducers was performed 24 hr before virus or tumor cell inoculation and thereafter on alternate days or at 3 day intervals for 13–16 days. The route and timing is indicated in the text. This method of treatment has been shown to be effective in the previous studies with murine leukemia and sarcoma *in vitro* and *in vivo* (7–11).

Results. Comparative in vitro *inhibitory effect of IF, NDV, statolon, and poly I·poly C on focus formation by mouse sarcoma virus.* Table I shows the comparative inhibitory effect of interferon (1300 units/ml), NDV ($10^{8.0}$ PFU/ml), statolon (100 μg/ml), and poly I·poly C (100 μg/ml) on MSV focus formation. These data were obtained by averaging the results of two experiments. All four were effective in inhibition of the viral transforming activity. However, NDV and statolon were most effective. A greater inhibitory effect of NDV on MSV foci was achieved using higher titered NDV.

TABLE I. Inhibitory Effect of Interferon (IF), Newcastle Diseases Virus (NDV), Statolon and Poly I·Poly C on Focus Formation by Moloney Sarcoma Virus.[a]

Virus dilution	Treatment[b]	Foci/plate (av of 2–3 plates)
0.5×10^{-1}	Interferon	18
	NDV	5
	Statolon	1
	Poly I·poly C	11
	None	85

[a] Interferon (1300 units); NDV (10^8 PFU); statolon (100 μg); poly I·poly C (100 μg). These data were obtained by averaging the titers of two experiments.

[b] Pretreatment, 24 hr.

TABLE II. Inhibitory Effect of Various Concentrations of Mouse Interferon[a] on Focus Formation by Moloney Sarcoma Virus.

Conc of interferon (units/ml)	Av no.[b] of foci/plate	Inhibition (%)
2500	4.3	93
1250	8.3	87
625	26.0	59
None	63.7	—

[a] Pretreatment, 24 hr.

[b] Average of 3 plates.

In previous studies (10, 11), we have shown that the effect was maximal with 100 μg of statolon or poly I·poly C/ml in NIH-METC pretreated for 24 hr. Treatment for 24 hr with higher concentrations of statolon and poly I·poly C was toxic for mouse embryo cells. Therefore, the doses of inducers which were used were close to their practical maximum.

In vitro *inhibitory effect of various concentrations of mouse IF on MSV foci.* The effect of varying the concentration of mouse IF on inhibition of sarcoma virus focus formation is shown in Table II. As shown in Table II, the extent of inhibition of MSV foci depends on the dose of IF used in the experiment. This is consistent with the observation that continued exposure of cells to IF exerts a greater inhibition of MSV than does a 24-hr pretreatment (8). Significant inhibition of foci was achieved with 1250 units or more of IF in NIH-METC pretreated for 24 hr. These findings indicate that *in vitro* all the inducers used and IF can exert a maximal inhibitory effect on transformation by MSV.

Comparative in vivo *inhibitory effect of IF and IF inducers (NDV, statolon, poly I·po-*

TABLE III. Inhibitory Effect of Interferon and Newcastle Disease Virus (NDV), Statolon, and Poly I·Poly C on the Induction of MSV-Induced Tumor in Newborn NIH Swiss Mice.[a]

Treatment	Proportion with tumors on days					
	8–10	11–13	14–16	(%)	24–26	(%)
IF	5/18	16/18	17/18	95[b]	18/18	100
NDV	1/18	5/18	7/18	39	13/18	72
Statolon	2/19	11/19	11/19	58	15/19	79
Poly I·poly C	1/15	8/15	9/15	60	12/15	80
HBSS	4/17	14/17	17/17	100	17/17	100

[a] IF, 200 units; NDV, 10^7 PFU; statolon, 100 μg; poly I·poly C, 100 μg; IF and IF inducers were given subcutaneously 24 hr before virus inoculation and at 2–3-day intervals for 16 days; HBSS = Hanks' balanced salt solution.

[b] Tumor incidence.

ly C) on MSV-induced tumor in mice. Table III shows the effect of IF and IF inducers administered locally (into the site of virus inoculation) on the induction of MSV tumors in NIH newborn Swiss mice. NDV, statolon, and poly I·poly C were equally effective. However, IF (200 units/mouse) was not effective. In control mice, tumors started to appear at the site of inoculation in 8 to 10 days and were observed in all the inoculated mice in 16 days. However, 40 to 61% of mice treated with poly I·poly C, statolon, and NDV failed to develop tumors. But 20 to 28% of the mice treated with IF inducers remained free of tumors at the end of observation period (26 days). The failure to obtain a response with IF might have been attributable to the low dose used. This possibility was studied next.

Effect of continued daily treatment with IF and NDV on MSV-induced tumor in mice. Figure 1 shows the effect of continued, daily, local treatment for 13 days with a higher dose of IF (5000 units/mouse), or NDV on MSV-induced tumors in NIH Swiss mice. Two different doses of MSV were used. As shown in Fig. 1, NDV was effective, however, 5000 units of mouse IF resulted in only a slight delay of appearance of tumors. The low effectiveness of IF on MSV in mice may be due to the relatively low dose of IF used in our experiment, since Gressor *et al.* (19, 20), Levy and Adamson (personal communication) achieved significant antitumor effect with IF using 10,000 units or more/mouse and daily treatment of several different tumors. A comparison of the doses of IF used and that generated by the inducers was examined next.

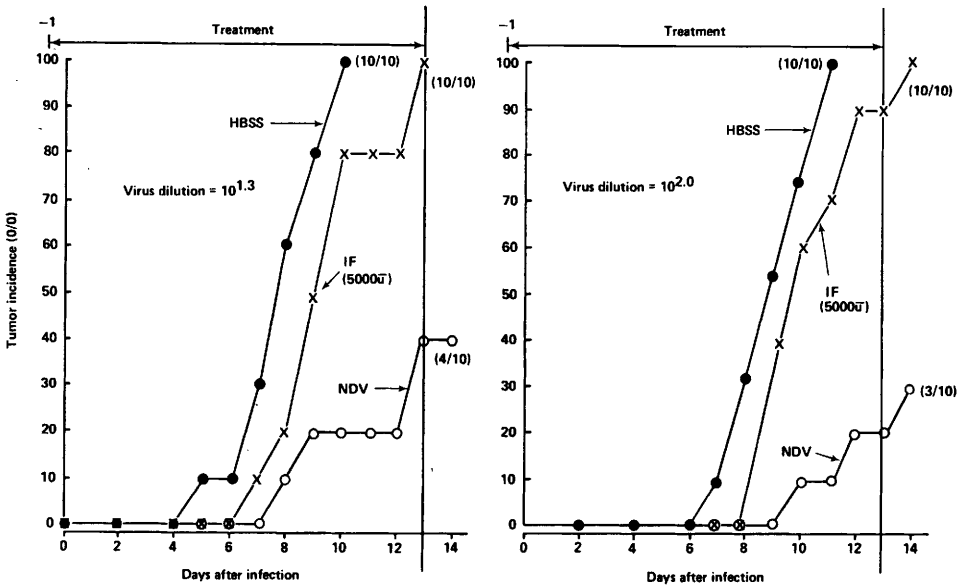

FIG. 1 Effect of continued daily treatment for 13 days with interferon (5000 units/mouse) or NDV on MSV-induced tumors in newborn NIH-Swiss mice.

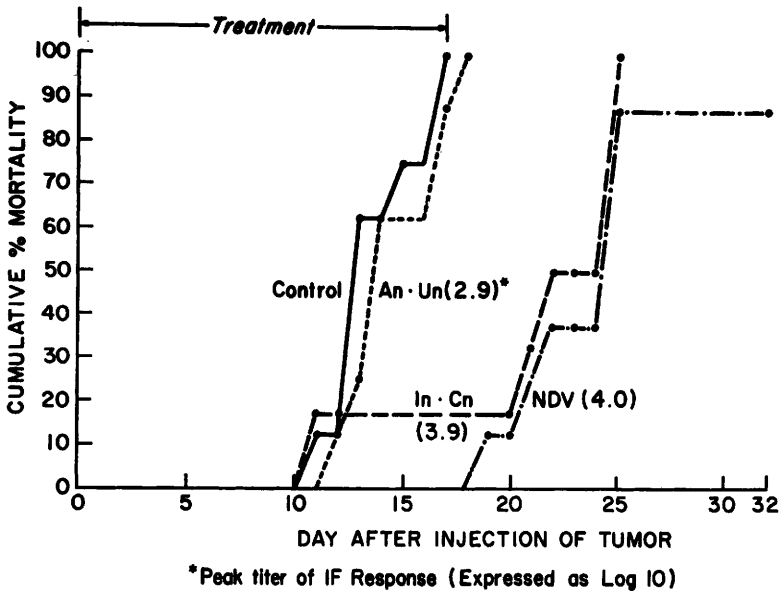

FIG. 2. Interferon response in the serum of mice following intraperitoneal injection of NDV, poly I·poly C, (In·Cn) and poly A·poly U (An·Un) and their antitumor effect on mouse Ehrlich ascites tumor (EAC_2H).

Interferon response in the serum of mice following injection of NDV, poly I·poly C and poly A·poly U and their antitumor effect on mouse Ehrlich ascites tumor, EAC_2H. Figure 2 shows the antitumor effect and IF response in CDF_1 mice. Ascites (EAC_2H) bearing mice were treated intraperitoneally with 200 μg of poly I·poly C, 200 μg of poly A·poly U, or 0.2 ml of NDV for 15 days (3×/week) beginning 24 hr after injection of tumor cells. IF response in the serum of CDF_1 mice following injection of NDV, poly I·poly C, and poly A·poly U are shown in Table IV. As shown in Fig. 2, greatest protection followed treatment with poly I·poly C or NDV. Poly A·poly U demonstrated no antitumor activity. The highest IF levels are stimulated by poly I·poly C and NDV. In contrast, poly A·poly U stimulated 1/10 as with IF.

TABLE IV. Interferon Response in the Serum of CDF_1 Mice Following Injection of NDV, Poly I·Poly C, and Poly A·Poly U.

Inducer	Route of injection[a]	Time (hr)	Interferon titers[b]
NDV	iv	6	4.0
	iv	24	2.2
Poly I·poly C	ip	4	3.9
	ip	24	3.1
Poly A·poly U	ip	4	2.9
	ip	24	1.7

[a] ip = intraperitoneal; iv = intravenous.
[b] Express as $\log_{10}$.

Effect of poly I·poly C and poly A·poly U on transplantability of Rauscher leukemia virus-induced tumors in hamsters. We examined the antitumor effect of poly I·poly C and poly A·poly U in the hamster system. Table V shows the effect of poly I·poly C and poly A·poly U on transplantability of virus-induced tumors in newborn hamsters. Poly I·poly C (25 μg) demonstrated some degree of antitumor effects (delayed tumor appearance and decreased tumor incidence). However, poly A·poly U demonstrated no such effect. The poor response of poly I·poly C in hamster system may be due to the poor IF response to poly I·poly C of hamster cells. We found that the IF response to poly I·poly C of hamster cells *in vitro* was much less than that of mouse cells, in that the minimum stimulative dose of poly I·poly C for hamster cells is ≧ 100 μg/ml, while that for mouse cells is 10 μg/ml. Similarly *in vivo*

TABLE V. Effect of Poly I·Poly C and Poly A·Poly U on Transplantability of Tumors in Newborn Hamster.[a]

Donor cells	Treatment	Proportion with tumors on day					
		5	7		12		
			(%)		—[c]		
RHT-1[b]	PBS	35/41	41/41	100	41/41	1.5–2.4	
(5.3 × 10^4 cells/ hamster)	Poly I·poly C (10 μg)	5/6	6/6	100	6/6	1.2–2.5	
	Poly I·poly C (25 μg)	5/37	24/37	65	32/37	(86%)	0.5–1.7
	Poly A·poly U (25 μg)	5/7	7/7	100	6/6	1.5–2.4	

[a] Interferon inducers were injected subcutaneously before tumor transplantation, followed by repeated treatment on alternate days or 3-day intervals; treatment stopped on day 12.
[b] RHT-1 = hamster tumor cell line derived from RLV-transformed hamster embryo cells.
[c] Tumor size (cm).

injection of 100 μg of poly I·poly C in weanling hamsters resulted in little or no circulating IF.

Discussion and Summary. A comparison of the antitumor effect of IF and IF inducers (NDV, statolon, poly I·poly C, and poly A·poly U) was made *in vitro* and *in vivo*. *In vitro* three IF inducers (NDV, statolon, poly I·poly C) and IF were highly effective in inhibition of focus formation by MSV. In mice, NDV, statolon and poly I·poly C were equally effective as antitumor agents. IF and poly A·poly U at the dose level used were not effective. In hamsters, the antitumor effects of the inducers were much less pronounced than they were in mice.

The present findings suggest that *in vivo* the antitumor effects of IF inducers may bear a relationship to the amount of IF induced. In mice, poly A·poly U is a much poorer inducer of IF and a much weaker antitumor agent than poly I·poly C, NDV, or statolon. The lack of significant antitumor effect of passive IF in mice might be attributable to the dose used, which was even less than the amount of IF induced by poly A·poly U. Under other conditions IF can manifest antitumor activity (19–20).

Further correlation of IF and antitumor effects comes from the finding that poly I·poly C is a much poorer inducer of IF and a much weaker antitumor agent in hamsters than it is in mice. Although these correlations are consistent with the interpretation that IF is one of the mediators of the antitumor effects of IF inducers (4–7, 11), other mechanisms of their action are not excluded (12, 21). For example poly I·poly C could also exert an antitumor action by enhancing the cell-mediated immune response (21, 22) but comparative studies of enhancement of cell immunity are not available for NDV, poly A·poly U, or statolon. Another possible mechanism which is not dealt with in the present study is a direct chemotherapeutic effect of the inducers (22). This effect has been shown for poly I·poly C, but comparison with the other inducers has not been reported.

Correlation between two phenomena, such as IF levels and antitumor effects, does not establish a causal relationship between them. Indeed under other circumstances, there was an apparent lack of correlation between serum IF levels and antitumor action (23).

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1. Gresser, I., Coppey, J., Falcoff, D., and Fontaine, D., C. R. Acad. Sci. **263**, 586 (1966).
2. Wheelock, E. F., Proc. Soc. Exp. Biol. Med. **124**, 855 (1967).
3. Youn, J. K., Barskin, G., and Huppert, J., C. R. Acad. Sci. Paris **267**, 816 (1968).
4. Levy, H. B., Law, L. W., and Rabson, A. S., Proc. Nat. Acad. Sci. U.S.A. **62**, 357 (1969).
5. Zeleznick, L. D., and Bhuyan, B. K., Proc. Soc. Exp. Biol. Med. **130**, 126 (1969).
6. Sarma, P. S., Shiu, G., Meubauer, R. H., Baron, S., and Huebner, R. J., Proc. Nat. Acad. Sci. U.S.A. **62**, 1046 (1969).
7. Sarma, P. S., Shiu, G., Baron, S., and Huebner,

R. J., Nature (London) **223**, 845 (1969).

8. Sarma, P. S., Baron, S., and Huebner, R. J., Nature (London) **224**, 604 (1969).

9. Rhim, J. S., Greenawalt, C., and Huebner, R. J., Nature (London) **222**, 1166 (1969).

10. Rhim, J. S., Huebner, R. J., and Gisin, S., Proc. Soc. Exp. Biol. Med. **130**, 181 (1969).

11. Rhim, J. S., and Huebner, R. J., Antimicrob. Ag. Chemother. **1969**, 177.

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

13. Kleinschmidt, W. J., Cline, J. C., and Murphy, E. B., Proc. Nat. Acad. Sci. U.S.A. **52**, 741 (1964).

14. Moloney, J. B., J. Nat. Cancer Inst. **16**, 877 (1956).

15. Hartley, J. W., and Rowe, W. P., Proc. Nat. Acad. Sci. U.S.A. **58**, 780 (1966).

16. Hartley, J. W., Rowe, W. P., Capps, W. I., and Huebner, R. J., Proc. Nat. Acad. Sci. U.S.A. **53**, 931 (1965).

17. Rhim, J. S., Huebner, R. J., Ting, R. C., Wivel, N., and Vass, W., Int. J. Cancer **5**, 28 (1970).

18. Baron, S., and Buckler, C. E., Science **141**, 1061 (1963).

19. Gresser, I., and Bourali, C., Nature (London) **223**, 844 (1969).

20. Gresser, I., Bourali, C., Levy, J. P., Fontaine-Brouty-Boye, D., and Thomas, M. T., Proc. Nat. Acad. Sci. U.S.A. **63**, 51 (1969).

21. Braun, W., and Nakano, M., Science **157**, 819 (1970).

22. Levy, H. B., Arch. Intern. Med. **126**, 78 (1970).

23. Weinstein, A., Gazdar, A., and Levy, H. B., Nature (London), (1971), in press.

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