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Direct Action of Interferon and Inducers of Interferon on Tumor Cells in Athymic Nude Mice[1]

Yves Rivière[2] and Ara G. Hovanessian

Unité d'Oncologie Virale, Institut Pasteur, 25, rue du Docteur Roux, 75724 Paris Cédex 15, France

ABSTRACT

Two interferon-mediated enzyme activities, the protein kinase and pppA (2′p5′A)$_n$ synthetase (2-5A synthetase) were used to assess the presence and action of interferon on HeLa tumor cells in athymic nude mice. The protein kinase is manifested by the phosphorylation of endogenous proteins with a molecular weight of 67,000 and 72,000 in mouse and human cells, respectively. Treatment of HeLa tumor-bearing mice with mouse interferon (α and β) resulted in enhanced levels of 2-5A synthetase and protein kinase (M_r 67,000) activities in the spleen and lung while there were no apparent effects on HeLa cells. In these HeLa tumor cells of human origin, the 2-5A synthetase and protein kinase (M_r 72,000) activities were enhanced considerably only after treatment of mice with human fibroblastic (β) interferon. When HeLa tumor-bearing mice were given injections of polyadenylate-polyuridylate or with polyinosinylate-polycytidylate, then the 2-5A synthetase and the protein kinase activities were enhanced in tumor cells [protein kinase] as well as in the different tissues [protein (M_r 67,000) kinase] of mice since both mouse and human interferons were produced under these conditions. These results indicate a direct action of interferon on homologous tumor cells, and furthermore they indicate that tumor cells in an organism may themselves produce interferon and respond to their own interferon.

INTRODUCTION

Treatment of tumor cell cultures with interferon results in the inhibition of cell growth (2, 3). Such an effect is also observed in tumor-bearing mice treated with interferon leading to the regression of tumor and increasing the survival rate (2, 3). The antitumoral action of interferon is probably mediated by 2 mechanisms, directly on tumor cells or indirectly by the activation of the host's defense mechanisms, such as the natural killer cell system. Previously, the latter effect has been emphasized by several studies (3, 14). For example, Gresser *et al.* (3) have shown that (α and β) interferon inhibits the growth of interferon- (α and β) resistant L1210 leukemia cells in mice although the degree of protection is much less than that observed against the parental L1210 interferon-sensitive cells. In this system, therefore, the inhibitory action of interferon on the growth of L1210-resistant cells is mediated by the host since these cells do not respond to mouse ($\alpha + \beta$) interferon *in vitro* (3, 5). Recently, Reid *et al.* (14) have confirmed this indirect action of interferon on tumor cells by studies on the tumorigenicity of heterologous cells in the nude mice. Against these studies, here we suggest that, in addition to its inhibitory effect mediated by the host, a direct action of interferon on tumor cells cannot be neglected. For this purpose, we measured 2 interferon-mediated enzymes, 2-5A synthetase[3] and protein kinase. The protein kinase activity is manifested by the phosphorylation of an endogenous protein with a molecular weight of 67,000 in mouse cells (p67 kinase) or a protein with a molecular weight of 72,000 in human cells (p72 kinase) (4, 5, 13). We have previously shown that the level of 2-5A synthetase and the protein kinase activities in the different tissues of mice reflect the presence and action of interferon (7, 10, 11) and can be used as convenient markers for assessing low levels of circulating interferon (9, 15). By the use of these enzymes, therefore, we assessed the immediate response of the different tissues of mice as well as of the tumor toward treatment with interferon. The results obtained provide biochemical evidence to show that interferon interacts with tumor cells in an organism. This is in accord with previously reported observations on the inhibitory action of human interferon on human tumors in the nude mice (1, 14, 17).

MATERIALS AND METHODS

Materials. All radiochemicals were supplied by the Radiochemical Centre, Amersham, England. Poly(A)·poly(U) and poly(I)·poly(C) were purchased from P. L. Biochemicals, Milwaukee, Wis.

Mice. Congenitally athymic nude mice (*nu/nu*; 8- to 12-week-old males) with the Swiss background were from Iffa Credo, L'Arbresle, France. Mice were maintained in a pathogen-free environment.

Plasma Preparation. After ether anesthesia, mice were bled from the axillary vessels, and the blood was collected in polystyrene tubes containing heparin (100 units/ml; Choay, Paris, France) and aprotinin (100 units/ml; Zymofren; Specia, Paris, France) and left for 15 to 30 min at 4°. Plasma was collected after centrifugation (200 × *g*, 15 min) and stored at −80°.

Interferon Titration. Mouse or human interferon activity in the plasma of HeLa tumor-bearing mice given injections of poly(A)·poly(U) was measured by the cytopathic effect of vesicular stomatitis virus on mouse L-929 or human MRC5 cells, respectively. Mouse interferon shows no activity on human cells while human interferon shows 1 to 5% activity on mouse cells. One international unit (NIH unit) of mouse interferon was equivalent to 30 effective laboratory units. One NIH unit of human interferon was equivalent to 2 effective units. Acid treatment (24 hr; 4°) of mouse plasma was by addition of HCl (1 N) to lower the pH to 2. The samples were then neutralized with NaOH (1 N) before titration for interferon activity (16). Human β interferon had a specific activity of 10^6 units/mg of protein. Partially purified mouse (α and β) interferon was prepared by the induction of mouse L-929 cells with Newcastle disease virus, and its specific activity was 5×10^7 units/mg of protein (11).

Tissue Extracts. Frozen tissues were homogenized mechanically (Ultraturrax type TP 18/2; 20,000 rpm) in low-salt buffer [10 mM HEPES, pH 7.6-10 mM KCl, 2 mM magnesium acetate-7 mM 2-mercaptoethanol-

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[2] To whom requests for reprints should be addressed.

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[3] The abbreviations used are: 2-5A synthetase, pppA (2′p5′A)$_n$ synthetase; p67 kinase, protein with a molecular weight of 67,000; p72 kinase, protein with a molecular weight of 72,000; poly(A)·poly(U), polyadenylate-polyuridylate; poly(I)·poly(C), polyinosinylate-polycytidylate; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

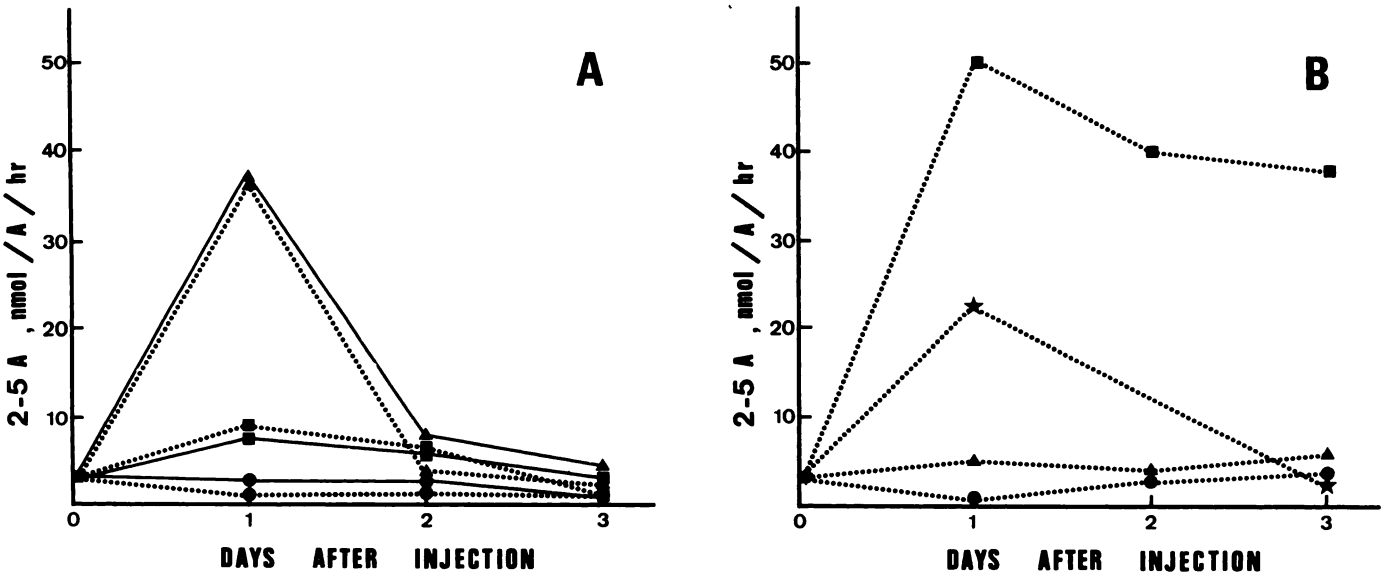

Chart 1. Response of normal and HeLa tumor-bearing nude mice to treatment with mouse and human interferon; level of 2-5A synthetase in the spleen (*A*) and in HeLa tumor cells (*B*). Athymic nude mice were given s.c. injections of HeLa cells (·····) or phosphate-buffered saline (——) and 3 weeks later were given i.v. injections (Day 0, *abscissa*) of 5×10^5 NIH units of human β interferon (■), 3.5×10^5 units of mouse (α and β) interferon (▲), or 0.9% NaCl solution (●). Injections were carried out twice in 0.2-ml volumes at 6-hr intervals. In a separate experiment (★), HeLa tumor-bearing mice were given i.v. injections of 0.2 ml of poly(A)·poly(U) solution (200 μg/mouse). Mice were sacrificed 1, 2, and 3 days after each treatment, and extracts from the different tissues were prepared. The level of 2-5A synthetase (*ordinate*) was measured as described in "Materials and Methods."

aprotinin (100 units/ml)]. This suspension was left for 15 min at 4° before addition of Nonidet P-40 at a final concentration of 0.5%. After 15 min, each suspension was sonicated for 10 sec and centrifuged at 1500 × *g* for 20 min (7). Tissue extracts were stored at −80°.

Assay of Protein Kinase. The p67 and p72 kinase activities were assayed after partial purification on poly(I)·poly(C)-Sepharose (9, 11). Phosphorylation (90 min; 30°) was with 10 nM [γ-^{32}P] ATP (6 Ci/mmol) in HEPES buffer-glycerol (10 nM HEPES, pH 7.6-50 mM KCl-5 mM magnesium acetate-10 mM $MnCl_2$-7 mM 2-mercaptoethanol-20% glycerol). Samples were heated in electrophoresis sample buffer and analyzed on polyacrylamide slab gels (10%) containing sodium dodecyl sulfate as described previously (8).

Assay of 2-5A Synthetase. Assay of 2-5A synthetase was in a total mixture (600 μl) containing: 200 μl of tissue extract (5 to 10 A_{260}); 20 mM HEPES, pH 7.6; 50 mM KCl; 25 mM magnesium acetate; 7 mM 2-mercaptoethanol; 5 mM ATP; 10 mM creatine phosphate; creatine kinase (0.16 mg/ml); poly(I)·poly(C) (0.1 mg/ml); and 2 μl of [^{3}H]ATP (1 mCi/ml; Amersham, England). Incubation was for 90 min at 30° and was terminated by heating at 90° for 5 min. ^{3}H-labeled 2-5A was purified as before (7), but the column was washed with buffer containing 50 mM KCl instead of 90 mM. The concentration of 2-5A in AMP equivalents was estimated from the percentage of incorporation of the radioactivity from input [^{3}H] ATP into 2-5A (^{3}H cpm). The 2-5A synthetase levels were calculated on this basis and are given as units corresponding to 1 nmol of 2-5A synthesized/1 unit A_{260}/hr (6, 13).

RESULTS

Response of Normal and HeLa Tumor-bearing Nude Mice to Treatment with Mouse and Human Interferon. The assay of 2-5A synthetase and the protein kinase was used to investigate the response of tumor cells to homologous and heterologous interferons. Athymic nude mice were given injections s.c. with HeLa cells (2×10^6), and 3 weeks later, when the tumor size had reached 10 mm in diameter, the different treatments were carried out as described. The level of 2-5A synthetase in the spleen and HeLa tumor cells (Chart 1) and the level of protein kinase activity (Table 1) (the p67 kinase in mouse organs and the p72 kinase in the HeLa cells) were measured in normal and HeLa tumor-bearing nude mice in the absence and presence of treatment with mouse (α and β) or human β interferon. These experiments were designed after *in vitro* studies which showed that HeLa cells do not respond to mouse interferon, and furthermore human β interferon has very little action (less than 1%) on mouse cells in contrast to human leukocytic (α) interferon which has some (5%) cross-reactivity.[4]

In accord with these *in vitro* observations, treatment of HeLa tumor-bearing mice with mouse interferon resulted in an enhanced level of 2-5A synthetase (Chart 1*A*) and p67 kinase (Table 1) in the spleen and the lung whereas no modification (2-5A synthetase and p72 kinase) was observed in HeLa tumor cells (Chart 1*B* and Table 1). On the other hand, treatment of similar mice with human β interferon led to an enhanced level of 2-5A synthetase and p72 kinase in HeLa tumor cells with a very slight effect in the spleen and the lung. The small enhancement in the level of enzyme activities in mouse tissues after treatment with human β interferon is probably due to the slight cross-reactivity (less than 1%) of human β interferon on mouse cells.

In these experiments, the protein kinase activity was measured after partial purification on poly(I)·poly(C)-Sepharose ("Materials and Methods"). The positions of the ^{32}P-labeled p67 (in the spleen and the lung) and p72 (in the HeLa tumor) were localized on the gel after electrophoresis, and small sections of the dried gels containing the radioactive bands were cut and counted (5). The level of the protein kinase activity was estimated, therefore, by the amount of $^{32}PO_4$ incorporated in the p67 and p72 (Table 1).

Response of HeLa Tumor-bearing Mice to Inducers of Interferon. HeLa tumor-bearing mice (as above) were given i.v.

[4] These results were obtained both by the assay of the antiviral response and by the level of 2-5A synthetase in mouse L-929 and human HeLa cells treated either with human α or β interferon or with mouse interferon (Y. Rivière and A. Hovanessian, unpublished observations).

Table 1

Level of protein kinase activity in the spleen, lung, and HeLa tumor of nude mice treated with human interferon, mouse interferon, or poly(A)·poly(U)

HeLa tumor-bearing nude mice were treated with 0.9% NaCl solution, human β interferon, mouse α + β interferon, or poly(A)·poly(U) as described in Chart 1. Twenty-four hr after each treatment, the protein kinase activity was assayed by the phosphorylation of p67 and p72 ("Materials and Methods"). Sections of gel containing the ^{32}P-labeled p67 and p72 were cut from the dried gels and counted in liquid scintillant (5).

Tissue	Treatment	^{32}P-labeled proteins (cpm) p67	p72
Spleen	NaCl	2,650	
	Human interferon	3,220	
	Mouse interferon	11,247	
	Poly(A)·poly(U)	9,591	
Lung	NaCl	4,375	
	Human interferon	4,986	
	Mouse interferon	15,966	
	Poly(A)·poly(U)	12,533	
Tumor	NaCl		3,261
	Human interferon		10,962
	Mouse interferon		3,085
	Poly(A)·poly(U)		8,725

injections with 200 μg of poly(A)·poly(U) or poly(I)·poly(C). The level of circulating interferon was then measured on mouse and human cells ("Materials and Methods"). In the plasma of both control and tumor-bearing mice, there were 80 and 1200 NIH units of mouse interferon 6 hr after injection with poly(A)·poly(U) and poly(I)·poly(C), respectively. In addition to mouse interferon, the plasma of tumor-bearing mice given injections of these synthetic double-stranded RNAs showed 30 and 120 NIH units of human interferon in response to poly(A)·poly(U) and poly(I)·poly(C), respectively. This human interferon was mostly acid (pH 2) stable. Plasma of control mice with or without injection of double-stranded RNA was devoid of interferon activity on human cells. These results were confirmed by the level of 2-5A synthetase and protein kinase in the lung, spleen, and HeLa cells of tumor-bearing mice. As expected, the level of 2-5A synthetase (data not shown; similar to Fig. 3 of Ref. 9) and p67 kinase were enhanced severalfold in the spleen and the lung (Table 1) of normal and tumor-bearing mice. Furthermore, in HeLa tumor cells, the level of 2-5A synthetase (Chart 1*B*) and p72 kinase (Table 1) was enhanced 24 hr after treatment with poly(A)·poly(U). Identical results were obtained with poly(I)·poly(C) as the interferon inducer (data not shown).

DISCUSSION

The results described here show that the level of 2-5A synthetase (Chart 1) and p67 kinase (data not shown) is comparable in the tissues of normal and tumor-bearing mice. On treatment with mouse interferon, these enzymes were enhanced at levels identical in both types of mice. Under these experimental conditions, therefore, the presence of tumor cells did not lead to the production of interferon nor trigger modifications in the response of the organism toward treatment with interferon. In HeLa tumor cells, the level of 2-5A synthetase and p72 kinase was affected by human β interferon but not by mouse interferon, thus indicating that interferon interacts directly with tumor cells which respond mainly to an interferon of similar species. HeLa tumor-bearing athymic nude mice were further investigated in relation to inducers of interferon such as poly(A)·poly(U) and poly(I)·poly(C). These double-stranded RNAs induced both mouse and human interferon in HeLa tumor-bearing mice. Accordingly, enhanced levels of 2-5A synthetase and the respective protein kinase activities were observed in mouse organs and HeLa tumor (p67 in the lung and spleen while p72 in HeLa tumor). Tumor cells stimulated by an inducer of interferon, therefore, produce interferon and then respond to their own interferon. These results show the advantage of interferon inducers over interferon in treatment of different tumors (8, 12), since the type of interferon used may not necessarily be the one which interacts with tumor cells (5).

The inhibitory effect of interferon on tumor cells in mice and in tissue culture is well documented. The precise mechanism of this antitumoral action, however, needs further investigation. Several authors have emphasized the role of interferon-mediated activation of the host's defense mechanisms. Gresser *et al.* (2, 3) have proposed a host-mediated effect in mice inoculated with interferon-resistant L1210 cells, and recently Reid *et al.* (14) have suggested that interferon may inhibit the growth of tumor cells by acting indirectly through components of the immune system such as natural killer cells. Here, we provided evidence for a direct action of interferon on tumor cells in athymic nude mice. By analogy with the *in vitro* anticellular effects of interferon, therefore, the overall action of interferon on tumor cells in an organism cannot be attributed only through its action on the host's defense mechanisms. There is also a direct interaction between interferon and tumor cells (Chart 1 and Table 1). Such an interaction is well illustrated by the enhanced levels of 2–5 synthetase and p72 kinase activities in HeLa tumor cells of tumor-bearing mice treated with human β interferon. The precise role of these enzymes in mediating the inhibitory action of interferon on tumor cells remains to be shown. The detection of these enzymes, however, serves as an efficient marker for the response of a tissue toward treatment with interferon.

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