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The Effect of Interferon on Cells Deficient in Nucleoside Transport or Lacking Thymidine Kinase Activity

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Mutants of mouse T-lymphoma S49 cells lacking thymidine kinase activity or deficient in nucleoside transport were selected by growth in the presence of 5'-fluorodeoxyuridine and their sensitivity to interferon tested. All five thymidine kinase and both transport deficient mutants were sensitive to the antiproliferative effects of interferon. The replication of encephalomyocarditis virus was also inhibited by interferon, and the intracellular levels of (2'-5')oligo-adenylate synthetase were elevated in all mutants tested. These results suggest that an intact nucleoside transport system or thymidine kinase activity are not essential for the expression of interferon sensitivity in these cells.

Interferons (IFNs) are a group of proteins which exert a number of specific effects on target cells, including inhibition of virus replication and cell growth and modulation of immune responses (1). The initial interaction of IFN with specific cell-surface receptors is necessary for expression of biological activity (2-4). The subsequent establishment of the antiviral state in treated cells requires RNA and protein synthesis (5) and involves induction of several proteins (6,7). Two of these interferon-induced proteins, 2'-5' oligo-adenylate (2-5A) synthetase (8-10) and eIF-2α protein kinase (11) are believed to be activated by viral dsRNA and mediate inhibition of virus replication through inhibition of protein synthesis (9-11). The mechanisms involved in the antiproliferative activi-

Abbreviations

Hu-IFN$\alpha_2\alpha_1$, human interferon $\alpha_2\alpha_1$; 2-5A, 2'-5' oligo-adenylate; eIF-2_α, eukaryotic protein synthesis initiation factor 2 (α subunit); dsRNA, double-stranded ribonucleic acid; DNA, deoxyribonucleic acid; TK, thymidine kinase or ATP: thymidine 5'-phosphotransferase (E.C.2.7.1.21); TK^-, thymidine kinase deficient mutant; T^-, nucleoside transport deficient mutant; EMC, encephalomyocarditis virus; PFV, plaque-forming units.

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ties of IFNs are much less understood. Significant changes in the rate of overall protein or RNA synthesis are not generally observed (12) but the incorporation of [^{3}H]-thymidine into DNA has been found to be depressed following interferon treatment (13-15). While this is often taken as a reflection of a slower rate of DNA synthesis in growth-inhibited cells, it has been previously shown that the transport and metabolic handling of the [^{3}H]-thymidine precursor can also be modified by interferons in a number of mouse and human cell systems (14-17). In the highly sensitive human lymphoblastoid (Daudi) cell line, reduced incorporation of [^{3}H]-thymidine is almost entirely due to changes in the membrane transport and phosphorylation of the precursor, while the absolute rate of DNA synthesis remains unaffected by interferon treatment (15,18). In mouse L-929 cells, sensitivity to interferon has been correlated with the presence of a functional thymidine kinase (TK) enzyme, TK deficient cells being resistant to interferon action (19,20). Taken together, these findings suggest that thymidine metabolism may be directly involved in mediating the effects of interferons on cell growth and viral replication, and in particular thymidine kinase may be essential for the expression of these activities. In the present paper, we describe variants of mouse T-lymphoma S49 cells which are lacking the TK enzyme (TK^-) and two mutants deficient in the membrane transport of nucleosides (T^-), all of which nevertheless retain their sensitivity to IFN.

MATERIALS AND METHODS

Materials

[^{3}H] Nitrobenzyl-6-thioinosine (16 ci/mmol) was purchased from ICN (Irvine, CA) and [Methyl-^{3}H] thymidine (5 ci/mmol) was purchased from New England Nuclear (Boston, MA).

Cell culture

Mouse T-lymphoma S49 cells were grown in Dulbecco's modified Eagles (DME) medium supplemented with either 10% horse serum or 5% foetal bovine serum. Thymidine kinase and nucleoside transport deficient mutants were selected for resistance to 5'-fluorodeoxyuridine (1 μM) and cells were cloned as previously described by Weinberg et al (21).

Thymidine kinase and nucleoside transport assays

Thymidine kinase activity was assayed in cell extracts using [^{3}H]-thymidine as substrate according to the procedure described by Cheng et al

(21). The number of nucleoside transport binding sites was determined by measuring the binding of radioactive nucleoside transport inhibitor [^{3}H]-nitrobenzylthioinosine as described in detail by Wiley et al (23).

Inhibition of cell growth by interferon

Duplicate aliquots of exponentially-growing cells (1-2x10^5/ml) were seeded in 24-well dishes (Falcon) and treated with interferon (Human IFN $\alpha_2\alpha_1$ (AD) kindly provided by Schering Corp) as indicated. Cells were counted in Coulter counter after further incubation at 37°C for 48 hrs.

2-5A synthetase assays

Exponentially-growing cells were treated for 12 hours with 0-500 u/ml IFN. The cells were washed once in phosphate buffered saline (4°C 1000 rpm, 15 mins) and pellets (10,000 xg, 1 min) stored at -70°C. Cell extracts were prepared and assayed for 2-5A synthetase as previously described (24).

Inhibition of virus replication

Exponentially growing cells were pre-treated for 12 hours with 100 u/ml interferon. The cells were washed once in fully supplemented growth medium (37°C) and incubated for a further 12 hours with encephalomyocarditis virus (EMC; 3 plaque forming units per cell). Virus yields were assayed by dilution of cytopathic effect of the supernatant medium on L-929 monolayers in 96-well microtiter plates (Nunclon).

RESULTS AND DISCUSSION

Seven 5-fluorodeoxyuridine resistant mutants of mouse T-lymphoma S49 cells were shown to exhibit less than 2% the exogenous thymidine incorporation of the wild-type parent S49 cells (Table 1). Five of these mutant cell lines were characterized as TK$^-$ (TK$^-$A, TK$^-$B, TK$^-$0, TK$^-$8, TK$^-$9) based on enzyme assays in cell-free extracts, while the two remaining mutants (T$^-$I4, T$^-$42) were found to be deficient in the binding of [^{3}H] nitrobenzylthioinosine and were thus defined as nucleoside transport negative (Table 1).

The response of the S49 cells and the mutants to the antiviral and anti-growth effects of interferons was tested using the recombinant DNA-derived human α-interferon $\alpha_2\alpha_1$ (Hu-IFN$\alpha_2\alpha_1$), a hybrid molecule which has been shown to be highly active on murine cells (25). The sensitivity of wild type S49 cells to IFN is comparable to that observed in other mouse cell systems. The wild-type and mutant cells were all sensitive to the anti-proliferative action of Hu-IFN$\alpha_2\alpha_1$ (Table 2), the TK mutants showing a comparable response to that of the wild-type cells, while transport deficient cells have a somewhat reduced response to interferon at the lower interferon concentrations.

Table 1

clone	[^{3}H] thymidine incorporation (cpm)	thymidine kinase activity (pmoles/10^6 cells/hr)	nucleoside transport sites (sites/cellx10^{-4})
Wild-type S49	160,200	1.81	6.5
TK$^-$A	2,990	<0.05	ND
TK$^-$B	2,220	<0.05	ND
TK$^-$O	1,270	<0.05	ND
TK$^-$8	1,240	<0.05	ND
TK$^-$9	2,610	<0.05	ND
T$^-$14	420	ND	<0.1
T$^-$42	200	ND	<0.1

[^{3}H] thymidine incorporation into DNA was estimated in microtiter trays after incubation of $1x10^5$ cells for 4 hrs in DME medium in the presence of 1% horse serum and 1 µci [^{3}H] thymidine (5 ci/mmol).

Thymidine kinase activity in cell extracts was assayed in triplicate samples as described in "Methods".

The number of nucleoside transport sites was estimated in duplicate samples of intact cells by assaying the number of sites which bind [^{3}H] nitrobenzylthioinosine under saturating conditions (10 mM) as described by Wiley et al (23).

The sensitivity of the cells to the antiviral actions of interferon was also tested. These effects are generally expressed at lower interferon doses and are characterized by protection of cells against viral infection and an increase in the intracellular levels of 2-5A synthetase. The TK$^-$

Table 2

Cell growth inhibition by HuIFN-$\alpha_2\alpha_1$.

Cells	Cell growth inhibition (% of control) 500 u/ml	1000 u/ml
Wild Type S49	28	29
TK$^-$A	34	40
TK$^-$B	27	34
TK$^-$O	17	25
TK$^-$8	36	35
TK$^-$9	24	28
T$^-$14	14	20
T$^-$42	14	26

Duplicate aliquots of exponentially growing cells were seeded in 24-well dishes and incubated for 48 hours with 500 or 1000 u/ml HuIFN-$\alpha_2\alpha_1$. Cells were counted in a Coulter Counter and growth inhibition expressed as a percentage of untreated cultures.

mutants of S49 cells all had elevated levels of this enzyme after interferon treatment, and the levels were several-fold higher than wild-type cells (Fig. 1A and B). Transport deficient mutants were also inducible for 2-5A synthetase (Fig. 1C). As in the case of growth inhibition, the T^- mutants were less responsive to low IFN concentrations (less than 200 u/ml) when compared to TK^- cells. The maximal level of induction of the enzyme was, however, similar for both types of mutants while being higher than wild-type cells.

EMC infection of cells pre-treated with 100 u/ml interferon resulted in an inhibition in the virus yield in all cases (Table 3). All the cells were sensitive, with a 4- to 32-fold reduction in virus yield, though there was no clear correlation between stimulation of 2-5A synthetase and reduction of virus yield (Table 3).

A number of previous reports have shown that interferons can modulate various aspects of thymidine metabolism, and that sensitivity to interferon may require the presence of active thymidine kinase. The data presented in this paper suggests that at least in some cell systems, this may not be the

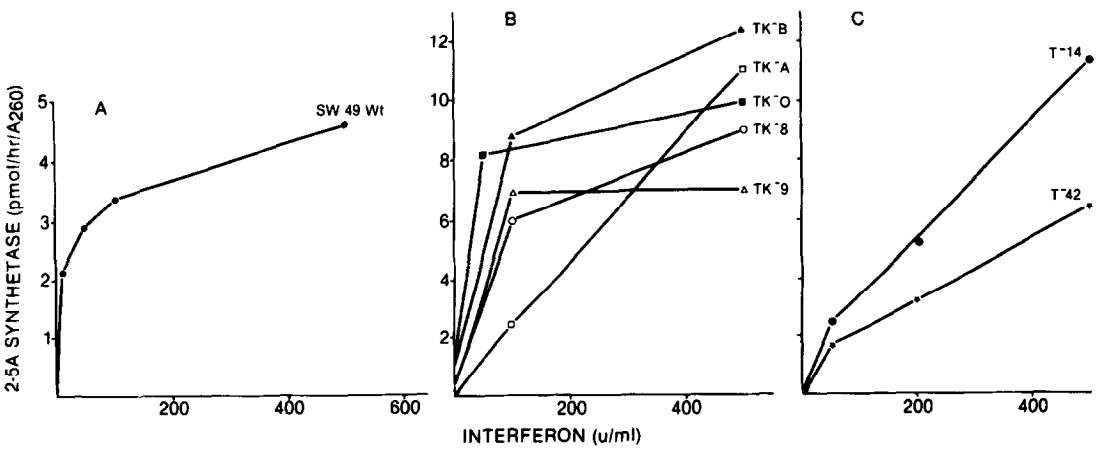

FIGURE 1 INDUCTION OF 2-5A SYNTHETASE BY HuIFN-$\alpha_2\alpha_1$

Duplicate aliquots of exponentially growing WT(A) TK^-(B) or T^-(C) cells were incubated with indicated amounts of interferon in DME medium supplmented with 1% foetal calf serum. After 12 hrs at 37°C, cells were centrifuged (1000 rpm, 10') and washed with 1 ml phosphate buffered saline before pelleting and freezing the cells at -70°C. Extracts were prepared and 2-5A synthetase assayed as described in "Methods".

Table 3

2-5A synthetase stimulation by Hu-IFN-$\alpha_2\alpha_1$ and reduction in virus yield.

	2-5A synthetase fold-stimulation	virus yield (C.P.E.$_{50}$ units/ml)	
	100 u/ml	Control	IFN-treated (100 u/ml)
Wild-Type S49	3.4	64	4
TK$^-$A	2.4	64	16
TK$^-$B	8.8	128	8
TK$^-$0	8.4	32	4
TK$^-$8	6	128	4
TK$^-$9	6.9	64	16
T$^-$14	3.4	128	16
T$^-$42	2.2	32	16

Exponentially growing cells were treated for 12 hours with 100 u/ml Hu-IFN-$\alpha_2\alpha_1$. 2-5A synthetase activity in cell extracts was measured as described in "Methods". Control and IFN-pretreated cells were incubated for 12 hours with EMC virus (3PFU/cell) and virus yield measured by dilution on L-929 monolayers in microtiter plates. Results are expressed as CPE$_{50}$ units per ml.

case. The five independantly isolated TK$^-$ mutants and two T$^-$ mutants of S49 cells described here, retain and may even express an enhanced sensitivity to both the antiviral and antiproliferative effects of interferon.

The presence of a functional thymidine kinase enzyme, or the ability to take up nucleosides from the medium, are therefore not required per se for the expression the interferon activity. However, it still remains possible that a secondary activity normally associated with the TK gene, in addition to the phosphorylation of thymidine, may be essential for interferon sensitivity (20). Thus a modification in the thymidine kinase gene product in TK$^-$S49 cells may have resulted in the loss of the thymidine phosphorylation activity without affecting the secondary activity. The isolation and characterization of further mutants may elucidate this activity.

In human lymphoblastoid (Daudi) cells, the marked inhibition of exogenous [^{3}H]-thymidine incorporation has been found to be almost entirely due to changes in the utilization of the precursor rather than in DNA synthesis

itself (15,18). Taken together with the present data, these findings suggest that modulation of thymidine incorporation may be only indirectly linked to interferon activity, possible as a consequence of the slower rate of cell growth, rather than as a mediator of interferon activity.

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