Dependence of the *in Vitro* Antiproliferative Activity of Recombinant Human γ-Interferon on the Concentration of Tryptophan in Culture Media

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ABSTRACT

In addition to its antiviral and antibacterial activities, recombinant human γ-interferon (rHuIFN-γ) can exert an antiproliferative effect on human cell lines. The mechanisms involved in this antiproliferative activity are poorly understood, but it is known that IFN-γ can induce indoleamine 2,3-dioxygenase, which enhances tryptophan metabolism and thus depletes the cellular pool of this amino acid. In the present study we have examined the effect of different tryptophan concentrations on the antiproliferative activity of rHuIFN-γ on four human tumor cell lines, HeLa 229, HEp-2, A549, and T24. Cells were grown in the presence of rHuIFN-γ (0.01 to 100 ng/ml) and/or tryptophan (10 to 400 μg/ml) for 7 days at which time they were counted. rHuIFN-γ (4 ng/ml) inhibited the growth of A549 and T24 cells by 50%. Hep-2 and HeLa 229 cells were more sensitive to the rHuIFN-γ induced antiproliferative effects, requiring only 0.4 ng/ml for a 50% inhibition. Addition of tryptophan to the media at concentrations from 10 to 100 μg/ml resulted in a significant blockage of the antiproliferative activity of rHuIFN-γ. For example, when 50 μg/ml of tryptophan were added to the media, 10 times more rHuIFN-γ (4 ng/ml) was needed to inhibit HeLa 229 cells by 50% of the control. The A549 was the most sensitive cell line to the modulatory activity of the tryptophan. Addition of 10 μg/ml of tryptophan changed the amount of rHuIFN-γ needed to produce a 50% inhibition from 4 ng/ml to 100 ng/ml. In summary, in the four human tumor cell lines tested, the antiproliferative activity of rHuIFN-γ could be modulated by the concentration of tryptophan in the media.

INTRODUCTION

The heterogeneous family of proteins termed interferons exert profound inhibitory effects on the growth of both normal and transformed cells (1). Similar to growth factors and hormones that affect cell proliferation, IFNs[1] must first bind to specific cell surface receptors and transmission of this signal from the receptor is necessary for activation of cellular genes and subsequent triggering of biological responses. While it is clear that IFN treatment results in the induction of specific cellular proteins, the roles of these proteins in the various effects of the different IFNs have not been clearly defined (2–4).

In 1978 Yoshida and Hayaishi (5) demonstrated that in mice, the i.p. injection of bacterial lipopolysaccharide resulted in the induction of pulmonary IDO. Consequently, investigators from the same laboratory were able to induce pulmonary IDO by infecting mice with influenza virus or by adding IFN-β to mouse lung slices (6, 7). More recently, Yasui *et al.* (8) have shown that human carcinoma lung tissues exhibited a significant increase in IDO activity resulting in the fast degradation of tryptophan to formylkynurenine. These authors concluded that IFN-γ mediated induction of IDO occurs in human lungs as a response to cancer and raised the hypothesis that depletion of tryptophan and accumulation of its degradation products may inhibit tumor growth.

In this report we have examined the growth of four human

[1] The abbreviations used are: IFN, interferon; IDO, indoleamine 2,3-dioxygenase; rHuIFN-γ, recombinant human γ-interferon; EMEM, Eagle's minimal essential medium; FBS, fetal bovine serum; TEA, triethylamine.

tumor cell lines which are sensitive to the antiproliferative activities of rHuIFN-γ and analyzed the modulatory effects of tryptophan on cell growth inhibition induced by this cytokine.

MATERIALS AND METHODS

Cell Lines. HEp-2 (a human epidermoid lung carcinoma cell line), HeLa 229 (a cell line from a cervical carcinoma), A549 (a human lung carcinoma cell line), and T24 (a cell line from a human bladder transitional cell carcinoma) cell lines were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in autoclavable EMEM supplemented with 5% FBS (Irvine Scientific, Irvine, CA) and gentamicin (50 μg/ml) and incubated at 37°C in a 5% CO₂ incubator.

rHuIFN-γ. rHuIFN-γ was a gift from Genentech, Inc. (South San Francisco, CA) (9, 10). The antiviral activity of rHuIFN-γ was determined in A549 cells challenged with encephalomyocarditis virus. All the experiments were performed with the same rHuIFN-γ preparation that had a specific activity of 3×10^7 IU/mg of protein. rHuIFN-γ was stored as a sterile solution at 4°C at concentrations ranging from 0.5 to 10 mg/ml in 20 mM Tris-HCl (pH 7.0) and 0.5 M NaCl.

Antiproliferative Activity. Cell monolayers were grown in tissue culture plates (24 wells) (Falcon, Oxnard, CA) by seeding the cells at a density of 1×10^4 cells/well. Twenty-four h later dilutions of rHuIFN-γ and/or tryptophan made in EMEM-5% FBS were added to the monolayers. Seven days later, just before control monolayers reached confluency, monolayers were washed with 0.01 M phosphate buffered saline (pH 7.4), trypsinized, and resuspended in Isotonic-II (Coulter Electronics, Inc., Hialeah, FL). The cells were counted using an automated cell counter (Model D2N Coulter Counter; Coulter Electronics, Inc.).

Amino Acids. Stock solutions of amino acids (L-tryptophan, L-aspartic acid, L-methionine, and L-lysine; Sigma Chemical Co., St. Louis, MO) were prepared in EMEM-5% FBS. Stock solutions (10 mg/ml) were filtered through a 0.2-μm filter (Gelman Scientific Instruments, Ann Arbor, MI) and stored at −20°C until used.

Analysis of Amino Acids Using Precolumn Derivatization with Phenylisothiocyanate. The method used was based on the formation of a phenylthiocarbamyl derivative of amino acids (11–13). Tissue culture fluid (60 μl) was incubated on ice with 240 μl of methanol for 15 min. DL-2-Aminobutyric acid was added to each sample as an internal control. The samples were then centrifuged for 15 min at 14,000 × g and 100-μl samples were used for analysis. The samples were dried on a speed vacuum concentrator and then resuspended in 25 μl of ethanol, water, and TEA. Derivatization was performed by adding 25 μl of ethanol-TEA-H₂O-phenylthiocarbamyl (7:1:1:1) to each sample and incubating at room temperature for 20 min. The samples were then dried and stored at −20°C until analyzed.

Reverse-phase high performance liquid chromatography was performed at 38°C with a Model 850 Dupont liquid chromatograph with a fixed wavelength Waters detector. The data were processed on a Shimadzu CR3A connected to a floppy disk drive.

The samples were dissolved in 100 μl of 0.05 M sodium phosphate buffer, pH 7.5, and then 10 μl were sandwiched between starting buffer and applied to the column in a 50-μl loop. Two Picotag columns (Waters) in tandem were eluted with a gradient from 11% eluent B to 51% eluent B in 33 min. Eluent A consisted of 0.14 M sodium acetate, 0.05% (v/v) TEA (pH 6.35), and eluent B consisted of 60% acetonitrile in water. The samples were monitored at a wavelength of 254 nm at a flow rate of 0.8 ml/min.

Statistical Analysis. Statistical significance was assessed by Student's *t* test.

RESULTS

Antiproliferative Activity of rHuIFN-γ. The antiproliferative activity of HuIFN-γ was tested by seeding cells and 24 h later adding fresh media (EMEM-5% FCS) containing rHuIFN-γ at concentrations ranging from 0.01 to 100 ng/ml. The control monolayer received media without rHuIFN-γ. Cells were grown for 7 days, the time necessary for control monolayers to reach 90–95% confluency. All results were expressed as a percentage of the number of cells found in the control, non-rHuIFN-γ treated monolayer.

Of the four cell lines tested, two of them, HeLa 229 and HEp-2, showed the greatest sensitivity to the inhibitory activity of rHuIFN-γ (Fig. 1). At concentrations of approximately 0.4 ng/ml of rHuIFN-γ there was a 50% growth inhibition of these two cell lines. At concentrations of 1–100 ng/ml of rHuIFN-γ, the number of HEp-2 cells was further decreased to approximately 20–25% of the control. The growth of the HeLa 229 cell line was greatly inhibited with only 16% of the cells remaining at 10 or 100 ng/ml of rHuIFN-γ. The other two cell lines tested, A549 and T24, were also susceptible to the inhibitory activity of rHuIFN-γ (Fig. 1). A 50% decrease in the proliferative activity was obtained at concentrations of approximately 4 ng/ml of IFN. Increasing the rHuIFN-γ concentration up to 100 ng/ml decreased the cell count to approximately 30% of the control.

Effects of Tryptophan on Cell Growth. To determine the effects of increasing concentrations of tryptophan on cell growth, the four cell lines were seeded; 24 h later the media were removed and fresh EMEM-5% FBS containing different concentrations of tryptophan, 10–400 µg/ml, were added. Control monolayers were fed with EMEM-5% FBS. Of the four cell lines tested, HEp-2 was the most sensitive to the antiproliferative effects of tryptophan (Fig. 2). Addition of 200 µg/ml of tryptophan decreased cell growth to 50% of the control, and addition of 400 µg/ml of tryptophan resulted in an inhibition of cell growth to 9% of the control monolayer. Concentrations

Fig. 2. Effect of tryptophan on cell proliferation. Cells were plated at low density (5 × 10³ cells/cm²) in EMEM-5% FBS and 24 h later, different concentrations of tryptophan (10, 25, 50, 100, 200, 300, or 400 µg/ml) were added to the media. Following 7 days of incubation, the cells were counted. The results are expressed as a percentage of the control (cells grown in EMEM-5% FBS). Each *point* represents the mean of three independent experiments.

of tryptophan from 10 to 400 µg/ml modified the growth of HeLa 229 cells, and at a concentration of 400 µg/ml of tryptophan, an inhibition of 50% of cell growth was obtained. Addition of increasing concentrations of tryptophan affected the growth of A549 and T24 cells to an equivalent degree. Addition of up to 100 µg/ml of tryptophan resulted in no significant alteration of the proliferative activity of these cell lines. A 20% inhibition was obtained at 200 µg/ml, and at 400 µg/ml approximately 40% inhibition was obtained in relation to control monolayers.

Combined Effects of rHuIFN-γ and Tryptophan. To determine the combined effects of rHuIFN-γ and tryptophan on cell growth, the two components were added simultaneously to the cells at concentrations ranging from 0.01 to 100 ng/ml of rHuIFN-γ, and from 10 to 400 µg/ml of tryptophan. Of the four cell lines tested, A549 appeared to be the most sensitive to the modulatory activity of tryptophan on the antiproliferative activity of rHuIFN-γ. As shown in Fig. 3, addition of only 10 µg/ml of tryptophan to the culture media significantly decreased the antiproliferative activity of rHuIFN-γ. For example, cells grown in media containing approximately 4 ng/ml rHuIFN-γ, but without additional tryptophan, were inhibited to 50% of the control. However, when 10 µg/ml of tryptophan were added simultaneously with rHuIFN-γ, a 50% inhibition of the growth of A549 cells required approximately 100 ng/ml of rHuIFN-γ. The maximum blockage of the antiproliferative activity of rHuIFN-γ was obtained in A549 cells when 25 µg/ml of tryptophan were added to the medium. rHuIFN-γ at concentrations of 100 and 10 ng/ml decreased cell growth to 26 and 31%, respectively, of the control in the regular EMEM-5% FBS; however, addition of 25 µg/ml of tryptophan resulted in a decrease of inhibition to 57 and 68% of the control, respectively (P < 0.0005). Similar results were obtained with the T24 cell
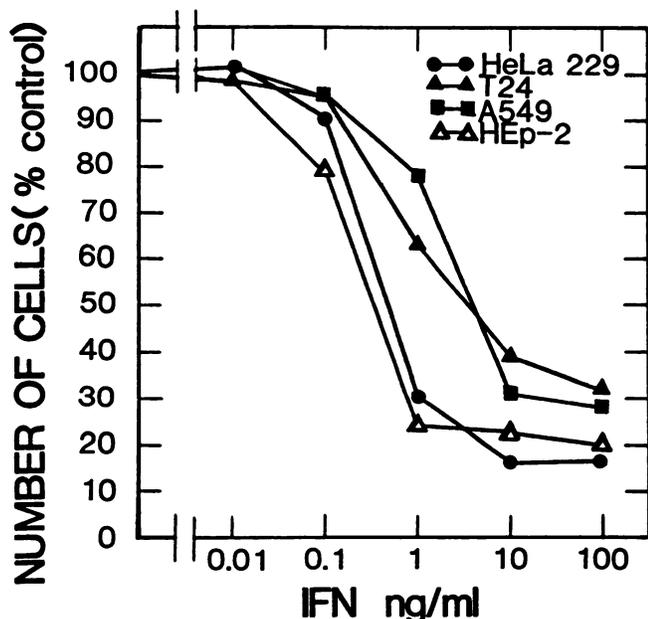
Fig. 1. rHuIFN-γ induced inhibition of cell growth. Cells were plated at low density (5 × 10³ cells/cm²) and 24 h postseeding different concentrations of rHuIFN-γ were added. After 7 days, the cells were counted. The results are expressed as a percentage of the number of cells in the control, non-rHuIFN-γ treated monolayers. Each *point* represents the mean of five independent experiments.
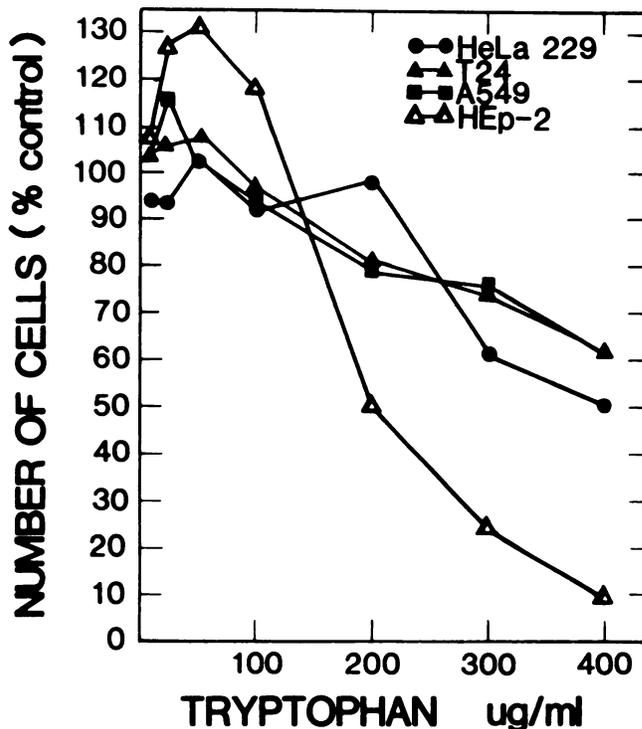
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line, although in this case, addition of higher concentrations of tryptophan (100 μg/ml) were required to obtain maximum blockage of the antiproliferative activity of rHuIFN-γ. Addition of 100 μg/ml of tryptophan rescued cell growth from 39 to 71% of the control at rHuIFN-γ concentrations of 10 ng/ml ($P <$ 0.0005).

Of the four cell lines tested, HEp-2 and HeLa 229 were the most sensitive to the antiproliferative activity of rHuIFN-γ, requiring approximately only 0.4 ng/ml of rHuIFN-γ in the media for a 50% inhibition (Figs. 1 and 3). The addition of tryptophan at concentrations of 50, 100, or 200 μg/ml to the media resulted in a shift of the 50% inhibition point of the HeLa 229 cells to approximately 3 ng/ml of rHuIFN-γ. At a concentration of 1 ng/ml of rHuIFN-γ, cell growth was 28% of the control in EMEM-5% FBS, but addition of 100 μg/ml of tryptophan to the media resulted in an increase in the proliferative activity of the cells to 60% of the control ($P <$ 0.0005).

Similar results were obtained with HEp-2 cells, but in this case, probably due to the higher sensitivity of these cells to the toxic effects of tryptophan, maximum protection was obtained with addition of 50 or 100 μg/ml of tryptophan. For example, at 10 and 1 ng/ml of rHuIFN-γ, significant differences were observed between cells grown in unsupplemented EMEM-5% FBS and those supplemented with 50 μg/ml of tryptophan. Cell growth was reduced to 22 and 24% of the control in the unsupplemented media, and addition of 50 μg/ml of tryptophan rescued the cells to 37 and 74% of the control ($P <$ 0.0005 and $P <$ 0.0025), respectively. At 200, 300, or 400 μg/ml of tryptophan there was again a decrease in proliferation of the cell line.

To further determine the effects due to tryptophan, we calculated the percentage rescue of cell growth by tryptophan using monolayers treated only with rHuIFN-γ as controls. In the case of the HeLa 229 or HEp-2 cells at a rHuIFN-γ concentration of 1 ng/ml, there was a 300 to 400% increase in the number of cells when 25, 50, or 100 μg/ml of tryptophan were added to the media. With A549 and T24 cells, the maximum rescue, on a percentage basis, was obtained at a rHuIFN-γ concentration of 10 ng/ml. In these two cell lines, addition of tryptophan at concentrations of 25, 50, or 100 μg/ml resulted in an increase of approximately 150 to 200% in the number of cells relative to the control monolayer treated with 10 ng/ml of rHuIFN-γ in EMEM-5% FBS.

Effect of Other Amino Acids on Cell Proliferation and on the Antiproliferative Activity of the rHuIFN-γ. To determine the specificity of the effect of tryptophan, three other amino acids, L-aspartic acid, L-methionine, and L-lysine, were tested for their ability to modify the growth of the four cell lines and to inhibit the antiproliferative activity of rHuIFN-γ. These three amino acids were tested by adding them to EMEM-5% FBS at concentrations of 100, 200, 300, or 400 μg/ml. None of the amino acids at any of the four concentrations tested resulted in a significant effect on cell proliferation (data not shown). Similarly, none of the three amino acids blocked the antiproliferative activity of rHuIFN-γ (100 ng/ml) (data not shown).

Effect of rHuIFN-γ on the Extracellular Pool of Amino Acids. Confluent monolayers were grown in EMEM-5% FBS or in EMEM-5% FBS with 10 ng/ml of rHuIFN-γ. Forty-eight h later the culture medium was analyzed using high performance liquid chromatography. The concentration of the following 12 amino acids was determined: L-arginine, L-glutamine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-threonine, L-tryptophan, L-tyrosine, and L-valine.
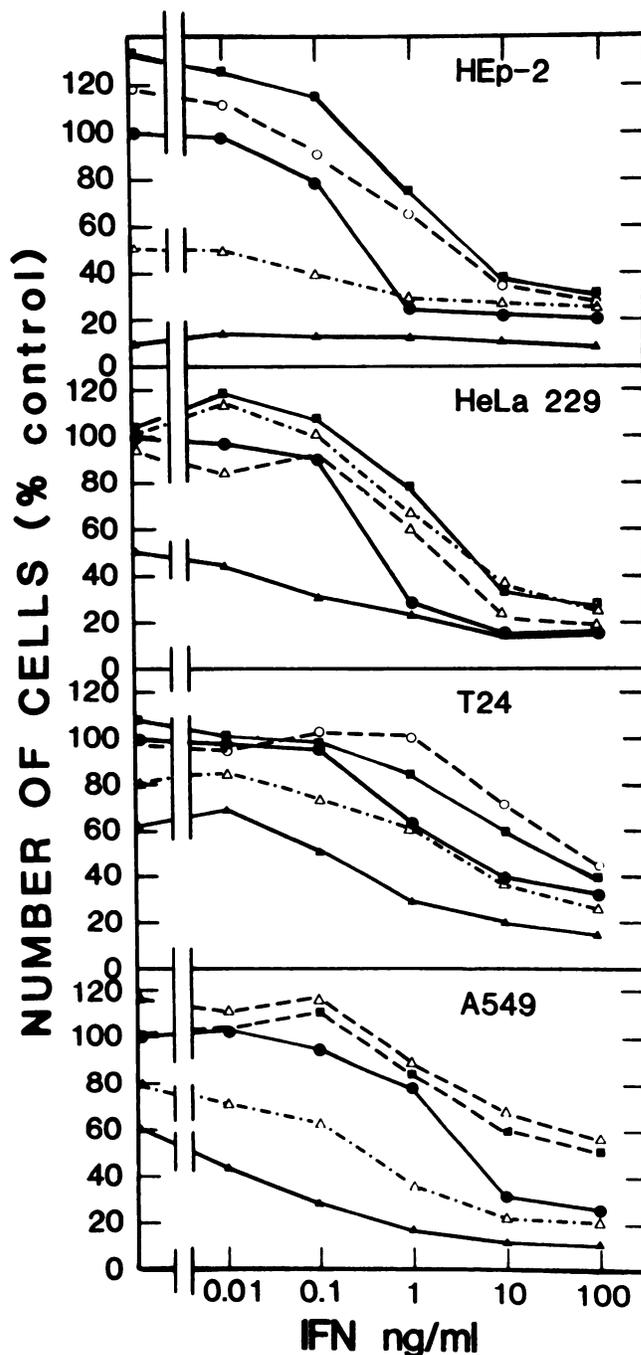

Fig. 3. Effect of rHuIFN-γ and tryptophan on cell proliferation. Monolayers were seeded at a density of 5×10^3 cells/cm^2 and 24 h later, media containing rHuIFN-γ and/or tryptophan were added. Cells were counted 7 days later and the number of cells was expressed as a percentage of the control (cells grown in EMEM-5% FBS). For each cell line, the control (●) and additional concentrations of tryptophan were tested. Tryptophan concentrations added: 10 μg/ml (■ - - ■); 25 μg/ml (Δ - - Δ); 50 μg/ml (■——■); 100 μg/ml (O); 200 μg/ml (Δ-·-Δ); 300 μg/ml (data not shown); 400 μg/ml (▲). In the figure, in addition to the control, only the tryptophan concentrations that resulted in the highest and the lowest inhibition and two intermediate concentrations are shown for each cell line. Each *point* represents the average of three independent experiments.

The percentage change in the rHuIFN-γ treated cells relative to the control culture was calculated for the four cell lines under investigation (Table 1). L-tryptophan was found to be the only amino acid significantly decreased in the media of the monolayers treated with rHuIFN-γ. The pool of extracellular tryptophan was decreased by approximately 60% (control 9.4 ± 0.5 μg/ml *versus* rHuIFN-γ treated cells 3.7 ± 0.4 μg/ml; $P <$ 0.005) in the rHuIFN-γ treated monolayers. In contrast, none

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Table 1 *Effect of rHuIFN-γ (10 ng/ml) on the extracellular amino acid pool*

The data are expressed as a ratio of the amino acid concentration in the rHuIFN-γ treated monolayer over the control monolayer. Each value represents the average of two independent experiments.

Amino acids	Cell lines			
	A549	HeLa 229	HEp-2	T-24
L-Arginine	0.92	1.04	0.94	1.14
L-Glutamine	0.89	0.96	1.01	0.90
L-Histidine	0.90	1.09	0.97	0.75
L-Isoleucine	0.90	1.14	1.04	1.03
L-Leucine	0.90	1.17	1.10	1.07
L-Lysine	0.91	0.87	0.97	1.38
L-Methionine	1.07	1.14	1.04	1.10
L-Phenylalanine	1.00	1.15	1.08	1.04
L-Threonine	0.99	1.03	1.07	1.12
L-Tryptophan	0.41	0.26	0.39	0.52
L-Tyrosine	1.00	1.13	1.05	1.04
L-Valine	0.96	1.17	1.08	1.06

of the other 11 amino acids tested shows a significant change in concentration when the rHuIFN-γ treated cells were compared to the controls.

DISCUSSION

The present study indicates that the amount of tryptophan present in the culture medium can modulate the *in vitro* antiproliferative activity of rHuIFN-γ on human tumor cell lines. In general, addition of tryptophan to EMEM-5% FBS in concentrations ranging from 25 to 100 μg/ml produced a significant blockage of the antiproliferative activity of rHuIFN-γ. By contrast, high concentrations of tryptophan in the media (200 to 400 μg/ml) resulted in a toxic effect on the cells, thus masking the blocking of the antiproliferative activity of rHuIFN-γ.

In 1981, Yoshida *et al.* (7) showed that IFN induced from murine fibroblasts or murine brain cells could produce a 10–15-fold increase in IDO in mouse lung slices. A group of investigators from the same laboratory had previously shown induction of IDO in mouse lungs following the i.p. injection of bacterial endotoxin or during an infection with influenza virus (5, 6). IDO is a hemoprotein that converts tryptophan to formylkynurenine by catalyzing the incorporation of superoxide anion or molecular oxygen into the pyrrole ring of tryptophan (14). The induction of IDO by IFN thus results in an increase in the catabolism of tryptophan reducing the pools of tryptophan available to the cell. Based on these findings, it became of great interest to study the effect of tryptophan on the antibacterial, antiviral, and antiproliferative activities of IFN.

In order to assess the effect of tryptophan on the inhibitory activity of IFN on the growth of intracellular microorganisms, the induction of IDO by IFN *in vitro* has been studied on several human cell lines, including HeLa 229, A549, and T24 (15–17). These investigators show that IFN could induce IDO in all of the human cell lines tested and that the induction of IDO resulted in an increase in degradation of tryptophan. Addition of tryptophan to the media rescues *Toxoplasma gondii* (15) and *Chlamydia psittaci* (17) but did not rescue *Rickettsia prowazekii* (16) from the IFN inhibitory effects. Furthermore, murine IFN-γ did not induce IDO in McCoy cells and thus tryptophan did not rescue *Chlamydia trachomatis* treated with mouse IFN-γ (16, 18). Similarly, data reported by Toth and Mecs (19) and Pfefferkorn *et al.* (20) suggested that, at least in some cell systems, tryptophan depletion does not play a major role in the antiviral activity of IFN-γ.

The tryptophan requirements for cell growth have been under investigation for several years. Deprivation of tryptophan in the culture medium was shown to result in an arrest of L1210

cell growth, and if the deprivation was extended for a period of three days, it resulted in the loss of viability of the cells (21). In our study, cell counts were done only at the end of the 7-day period. Thus, we do not know if the cell growth rate was influenced throughout the 7 days or if the antiproliferative effect occurred mainly late in the incubation period when there was a large number of cells and a significant depletion of extracellular tryptophan. The mechanisms by which tryptophan deprivation results in cell death are poorly understood, but they may be a consequence of the role that tryptophan plays as a precursor of proteins and coenzymes such as NAD, NADP, and poly(ADP-ribose) (22). The four human tumor cell lines that we tested, although of different origin, HEp-2 and A549 from the lung, T-24 from the bladder, and HeLa 229 from the cervix, all showed a blockage by tryptophan of the antiproliferative activity induced by rHuIFN-γ, thus suggesting that this might be a universal phenomenon in human tissues with *in vivo* implications.

In vivo evidence for a role of tryptophan in the regulation of cell growth was provided by showing that a decrease of the ingestion of tryptophan can reduce the growth of tumors in mice (23). Human *in vivo* data also support a role for tryptophan in the control of cell growth by IFN. An increase in the urinary excretion of tryptophan metabolites of the kynurenine pathways was described in patients with cancer or with infectious diseases (22). Furthermore, Byrne *et al.* (24) have shown that injection of rHuIFN-γ into cancer patients results in a decrease in tryptophan serum levels. Recently, Yasui *et al.* (8) demonstrated that lung tissues from humans with lung cancer have increased IDO activity and that rHuIFN-γ can induce this enzyme in human lung tissues. These authors proposed that an increase in pulmonary IDO activity in patients with lung cancer is a result of the induction by HuIFN-γ of this enzyme. They also suggest that as a result of the degradation of tryptophan, there is a depletion of the tryptophan pool leading to an inhibitory effect on tumor growth. Our results provide experimental evidence for this proposal and indicate that there is a fine balance between tryptophan concentration and the effect of IFN on cell growth. Our *in vitro* model should help to further investigate the mechanisms that are involved in this IFN-induced metabolic pathway.

ACKNOWLEDGMENTS

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