RESEARCH COMMUNICATION

4-Chloro-3-hydroxyanthranilate, 6-chlorotryptophan and norharmane attenuate quinolinic acid formation by interferon- γ -stimulated monocytes (THP-1 cells)

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Accumulation of quinolinic acid and L-kynurenine occurs in the brain and/or blood following immune activation, and may derive from L-tryptophan following induction of indoleamine 2,3-dioxygenase and other kynurenine-pathway enzymes. In the present study a survey of various cell lines derived from either brain or systemic tissues showed that, while all cells examined responded to interferon- γ by increased conversion of L-[$^{13}\text{C}_6$]tryptophan into L-kynurenine (human: B-lymphocytes, neuroblastoma, glioblastoma, lung, liver, kidney; rat brain: microglia, astrocytes and oligodendrocytes), only macrophage-derived cells (peripheral-blood mononuclear cells; THP-1, U-937) and certain liver cells (SKHep1) synthesized [$^{13}\text{C}_6$]quinolinic acid. Tumour necrosis factor- α enhanced the effects of interferon- γ in THP-1 cells. Norharmane, 6-chloro-DL-tryptophan and 4-chloro-3-hydroxyanthranilate attenuated quinolinic acid formation by THP-1 cells with IC_{50} values of 51 μM , 58 μM and

0.11 μM respectively. Norharmane and 6-chloro-DL-tryptophan attenuated L-kynurenine formation with IC_{50} values of 43 μM and 51 μM respectively, whereas 4-chloro-3-hydroxyanthranilate had no effect on L-kynurenine accumulation. The reductions in L-kynurenine and quinolinic acid formation are consistent with the reports that norharmane is an inhibitor of indoleamine 2,3-dioxygenase, 6-chloro-DL-tryptophan is metabolized through the kynurenine pathway, and 4-chloro-3-hydroxyanthranilate is an inhibitor of 3-hydroxyanthranilate 3,4-dioxygenase. These results suggest that many tissues may contribute to the production of L-kynurenine following indoleamine 2,3-dioxygenase induction and immune activation. Quinolinic acid may be directly synthesized from L-tryptophan in both macrophages and certain types of liver cells, although uptake of quinolinic acid precursors from blood may contribute to quinolinic acid synthesis in cells that cannot convert L-kynurenine into quinolinic acid.

INTRODUCTION

Quinolinic acid (QUIN) and L-kynurenine (L-KYN) are two neuroactive L-tryptophan metabolites and kynurenine-pathway metabolites [1]. Substantial accumulations of QUIN and L-KYN occur in the brain and blood of patients and non-human primates with a broad spectrum of inflammatory neurological diseases, particularly those with neurological impairments and inflammatory lesions within the central nervous system [2–5]. Because cerebrospinal fluid QUIN levels increase prior to, and in conjunction with, the development of neurological impairments, and because cerebrospinal-fluid QUIN concentrations correlate with the severity of neurological symptoms in patients infected with the human immunodeficiency virus (HIV) [4,6], we postulated that QUIN may be involved in the pathogenesis of neurological disturbances in inflammatory neurological diseases. If this hypothesis is correct, then strategies to reduce the synthesis of QUIN may have therapeutic benefit.

The increases in QUIN and L-KYN levels in brain and blood following immune activation are attributed to induction of indoleamine 2,3-dioxygenase, kynurenine 3-hydroxylase, kynureninase and 3-hydroxyanthranilate 3,4-dioxygenase [2–4,7–11]. Macrophages were proposed as an important, but not unique, localization for the production of QUIN [3,4,9,10]. The present study has surveyed a broad spectrum of human cell lines obtained from several tissues, as well as rat microglia, astrocytes and

oligodendrocytes, to determine which cells can convert L-tryptophan into L-KYN and QUIN when stimulated by interferon- γ (IFN- γ). 6-Chloro-DL-tryptophan has been reported to be metabolized through the kynurenine pathway to form 4-chloro-3-hydroxyanthranilate [12], an inhibitor of 3-hydroxyanthranilate 3,4-dioxygenase and QUIN synthesis [13]. Norharmane has been reported to be an inhibitor of indoleamine 2,3-dioxygenase [14]. The present study has examined whether 6-chloro-DL-tryptophan, 4-chloro-3-hydroxyanthranilate or norharmane can attenuate the formation of QUIN and L-KYN in monocytes stimulated with IFN- γ .

MATERIALS AND METHODS

Chemicals

L-KYN, QUIN, 1,1,1,3,3,3-hexafluoropropan-2-ol and Trypan Blue were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Trifluoroacetylhydrazide was acquired from Pierce Chemical Co. (Rockford, IL, U.S.A.). RPMI-1640, L-glutamine and fetal-bovine serum were obtained from Biofluids (Rockville, MD, U.S.A.). L-[ring- $^{13}\text{C}_6$]Tryptophan and [ring- $^{13}\text{C}_6$]anthranilic acid were acquired from MSD Isotopes (Montreal, Canada). Human and rat recombinant IFN- γ and human recombinant tumour necrosis factor- α (TNF- α) were obtained from Amgen Biologicals (Thousand Oaks, CA, U.S.A.). 6-Chloro-DL-tryptophan and 4-chloro-3-hydroxyanthranilate were generously given

Abbreviations used: IFN, interferon; L-KYN, L-kynurenine; QUIN, quinolinic acid; TNF- α , tumour necrosis factor- α .

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Table 1 L-KYN and [$^{13}\text{C}_6$]QUIN production from L-tryptophan by human peripheral-blood mononuclear cells, human cell lines and rat glial cells *in vitro* following stimulation with IFN- γ

Cells were incubated with 50 μM L- $^{13}\text{C}_6$ tryptophan either with or without (control) 100 i.u./ml IFN- γ . L-KYN (μM , labelled and unlabelled) and [$^{13}\text{C}_6$]QUIN (nM) concentrations after 48 h incubation are expressed as mean $\pm$ 1 S.E.M. for three incubation wells per time point. The minimum sensitivities of the L-KYN and [$^{13}\text{C}_6$]QUIN were 0.05 μM and 0.2 nM respectively. Note: baseline L-KYN levels in the serum added to the incubation media (approx. 0.05 μM) have been subtracted. The minimum detectable concentrations were: QUIN $\leq$ 0.2 nmol/l, L-KYN $\leq$ 0.05 μM . Abbreviation: N.D., not detected. For statistical comparisons, N.D. was set at the minimum detectable values; * $P < 0.01$ by the Kruskal–Wallis test.

Origin	Cell	Concn. (μM)			
		L-KYN		[$^{13}\text{C}_6$]QUIN	
		Control	IFN- γ	Control	IFN- γ
Peripheral-blood mononuclear cell	Macrophage	0.57 $\pm$ 0.05	37.9 $\pm$ 1.6	0.12 $\pm$ 0.003	10.47 $\pm$ 0.68*
	THP-1	0.21 $\pm$ 0.05	35.3 $\pm$ 3.2*	0.05 $\pm$ 0.001	1.71 $\pm$ 0.25*
	U937	N.D.	1.87 $\pm$ 0.12*	N.D.	0.06 $\pm$ 0.01*
B-Lymphocyte	WIL-NS	N.D.	0.24 $\pm$ 0.03*	N.D.	N.D.
Liver	SKHep1	N.D.	63.2 $\pm$ 0.46*	N.D.	0.24 $\pm$ 0.02*
	HepG2	N.D.	0.75 $\pm$ 0.01*	N.D.	N.D.
	MRC-9	N.D.	59.3 $\pm$ 2.7*	N.D.	N.D.
Lung	A-498	N.D.	43.2 $\pm$ 1.2*	N.D.	N.D.
Kidney	SK-N-SH	N.D.	33.6 $\pm$ 0.9*	N.D.	N.D.
Neuroblastoma	H4	9.8 $\pm$ 0.8	66.0 $\pm$ 5.2*	N.D.	N.D.
Neuroglioma	U373MG	1.50 $\pm$ 0.49	71.8 $\pm$ 1.4*	N.D.	N.D.
Astrocytoma/glioblastoma	Microglia	N.D.	0.78 $\pm$ 0.06*	N.D.	N.D.
	Astrocyte	N.D.	1.54 $\pm$ 0.08*	N.D.	N.D.
	Oligodendrocyte	N.D.	1.01 $\pm$ 0.07*	N.D.	N.D.

by Dr. J. Parli and the Eli Lilly Co., Indianapolis, IN, U.S.A. The remaining reagents were obtained from Fisher (Fair Lawn, NJ, U.S.A.).

Cell preparations and *in vitro* incubation conditions

Cells were incubated in media supplemented with 50 μM L- $^{13}\text{C}_6$ tryptophan, and either with or without IFN- γ (100 units/ml) and/or TNF- α (100 units/ml). Peripheral-blood macrophages from human volunteers were obtained from Advanced Biotechnologies (Columbia, MD, U.S.A.). Macrophages were incubated in a 75 cm^2 plastic culture flask at a density of 10^7 cells/flask in 20 ml of tissue-culture medium consisting of L-tryptophan-free RPMI-1640, 10% heat-inactivated fetal-bovine serum, 2 mM L-glutamine, 25 $\mu\text{g}/\text{ml}$ gentamicin, 100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin [9]. The different human cell lines used were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.; [15]). THP-1, U937 and WIL-NS cells were incubated in L-tryptophan-free medium containing RPMI-1640 [15]. The remaining cell lines were incubated in 10–20 ml of culture media whose composition was described previously [15] and which contains $\geq$ 50 μM L-tryptophan. Cells were incubated in 75 cm^2 plastic culture flask at a density of 10^5 – 10^6 cells/ml or 80% confluence. Primary cultures of microglia, astrocytes and oligodendrocytes were isolated and cultured from 2-day-old Sprague–Dawley rats by established methods [16,17]. All cells were cultured at 37°C in humidified air containing 5% CO_2 . To confirm cell survival and viability, the Trypan Blue exclusion test was used. After 24 and 48 h incubation the concentrations of L-KYN and [$^{13}\text{C}_6$]QUIN were quantified.

Quantification of L-KYN, QUIN and [$^{13}\text{C}_6$]QUIN

The concentrations of [$^{13}\text{C}_6$]QUIN released into the incubation medium (100 μl) was determined by g.c. and electron-capture negative-chemical-ionization m.s. as described previously [9]. The minimum sensitivity of the QUIN assay at a signal-to-noise

ratio of 10:1 was 0.0002 $\mu\text{mol}/\text{l}$. L-KYN was quantified by h.p.l.c. and u.v.-light-absorbance spectrometry [18]. The minimum sensitivity of the L-KYN assay at a signal-to-noise ratio of 10:1 was 0.05 μM .

Statistical analyses

Results were analysed by one-way analysis of variance, with Dunnett's *t* test as the *post hoc* test, or analysed by the Kruskal–Wallis test [19]. Regression analysis was done by the method of least squares. A *P* value of < 0.01 was considered statistically significant.

RESULTS

An initial survey of cell lines derived from both the central nervous system and systemic tissues showed that, while IFN- γ increased indoleamine 2,3-dioxygenase activity in all cell types examined, as demonstrated by the production of L-KYN (labelled and unlabelled) only cells which are derived from human monocytes (peripheral-blood mononuclear cells, THP-1 cells and U-937 cells), and SKHep1 liver cells were able to convert L- $^{13}\text{C}_6$ tryptophan into [$^{13}\text{C}_6$]QUIN (Table 1). The QUIN/L-KYN ratio was higher in peripheral-blood mononuclear cells (0.276) compared with THP-1 cells (0.048) and U-937 cells (0.032). No [$^{13}\text{C}_6$]QUIN was detected in incubation media of rat astrocytes, oligodendrocytes or microglia, despite clear induction of indoleamine 2,3-dioxygenase and synthesis of L-KYN (Table 1). Endogenous QUIN was detectable in all media, owing to the QUIN that was present in the serum. In cell incubations where [$^{13}\text{C}_6$]QUIN was not detectable (N.D., Table 1), endogenous QUIN levels were unchanged following incubation with IFN- γ (Table 1). This demonstrates that QUIN is not catabolized by these cells.

In THP-1 cells, TNF- α alone slightly increased L-KYN and [$^{13}\text{C}_6$]QUIN concentrations. TNF- α slightly enhanced L-KYN and [$^{13}\text{C}_6$]QUIN accumulations in response to IFN- γ (Table 2).

Table 2 L-KYN and [$^{13}\text{C}_6$]QUIN production from L-tryptophan by THP-1 cells *in vitro* following stimulation with IFN- γ

Cells were incubated with 50 μM L-[$^{13}\text{C}_6$]tryptophan, either with or without (control) IFN- γ (100 i.u./ml) or TNF- α (100 i.u./ml). L-KYN (μM , labelled and unlabelled) and [$^{13}\text{C}_6$]QUIN (μM) concentrations after 48 h incubation are expressed as mean $\pm$ 1 S.E.M. for three incubation wells. The minimum sensitivities of the L-KYN and [$^{13}\text{C}_6$]QUIN were 0.05 μM and 0.2 nM respectively. Note: baseline L-KYN levels in the serum added to the incubation media (approx. 0.05 μM) have been subtracted. Statistical significance: * P < 0.01 versus control; † P < 0.01 of IFN- γ + TNF- α alone or versus TNF- α alone by the Kruskal-Wallis test.

Source of stimulation	Concn.	
	L-KYN (μM)	QUIN (μM)
None (control)	0.28 $\pm$ 0.03	0.05 $\pm$ 0.02
IFN- γ (100 units/ml)	18.6 $\pm$ 0.72*	2.2 $\pm$ 0.3*
TNF- α (100 units/ml)	0.72 $\pm$ 0.71*	0.20 $\pm$ 0.15*
TNF- α (100 units/ml) IFN- γ (100 units/ml)	29.1 $\pm$ 1.10†	3.65 $\pm$ 0.03†

No [$^{13}\text{C}_6$]QUIN was detected in unstimulated or IFN- γ -stimulated THP-1 cells where [$^{13}\text{C}_6$]anthranilic acid (50 μM), a putative precursor of QUIN, was substituted for L-[$^{13}\text{C}_6$]tryptophan.

The effects of 6-chloro-DL-tryptophan, 4-chloro-3-hydroxyanthranilate and norharmane were studied in the THP-1 cell line. When added to the THP-1 incubations, both 6-chloro-DL-tryptophan and norharmane significantly attenuated the production of L-KYN (Figure 1a) and [$^{13}\text{C}_6$]QUIN (Figure 1b) in a dose- and time-dependent manner. The IC_{50} values of the inhibition of L-KYN formation were 51 μM for 6-chloro-DL-tryptophan and 43 μM for norharmane. The IC_{50} values of the inhibition of [$^{13}\text{C}_6$]QUIN formation were 58 μM for 6-chloro-DL-tryptophan and 51 μM for norharmane. 4-Chloro-3-hydroxyanthranilate also reduced the formation of [$^{13}\text{C}_6$]QUIN, with an IC_{50} value of 0.11 μM , but had no significant effect on the increased accumulation of L-KYN by IFN- γ treated THP-1 cells. In all studies of THP-1 cells, the Trypan Blue exclusion remained > 85% in control and drug-treated cultures. Dexamethasone and indomethacin at concentrations of 1 μM and 5 μM respectively had no effect on L-KYN or [$^{13}\text{C}_6$]QUIN accumulation by IFN- γ -stimulated THP-1 cells.

DISCUSSION

A highly specific and accurate m.s. assay was used to identify which cell types can convert L-[$^{13}\text{C}_6$]tryptophan into [$^{13}\text{C}_6$]QUIN when stimulated by IFN- γ . The effects of putative kynurenine-pathway inhibitors on QUIN and L-KYN formation were also studied.

The results (Table 1) show that IFN- γ increased L-KYN production in all cell types examined, presumably via induction of indoleamine 2,3-dioxygenase activity. It is likely, therefore, that many cell types and tissues are sources for L-KYN following systemic and intracerebral immune activation [3,4,10,11]. The ability of cells to convert L-tryptophan into QUIN, however, was not ubiquitous. Human monocytes (peripheral-blood mononuclear cells and THP-1 and U-937 cell lines) converted L-[$^{13}\text{C}_6$]tryptophan into [$^{13}\text{C}_6$]QUIN, and the rate of this formation was accelerated by IFN- γ . These observations are consistent with our previous reports [3,9], but contradict other findings [20]. One study has reported that TNF- α does not induce indoleamine 2,3-dioxygenase in mononuclear cells, but can potentiate the

Figure 1 Effects of 6-chloro-DL-tryptophan, norharmane and 4-chloro-3-hydroxyanthranilate on L-KYN and [$^{13}\text{C}_6$]QUIN production by IFN- γ -stimulated THP-1 cells

Cells were incubated with 50 μM L-[$^{13}\text{C}_6$]tryptophan, 100 i.u./ml IFN- γ and various concentrations (abscissa) of either 6-chloro-DL-tryptophan ($\circ$), norharmane ($\bullet$), 4-chloro-3-hydroxyanthranilate ($\square$) or no inhibitor (control). L-KYN (labelled and unlabelled) (a) and [$^{13}\text{C}_6$]QUIN (b) concentrations (ordinate) after 48 h incubation are expressed as the mean $\pm$ 1 S.E.M. (three incubation wells per point) and are percentages of the level in IFN- γ -stimulated cultures with no inhibitor added. Unstimulated: L-KYN, 0.24 $\pm$ 0.08 μM ; QUIN, 0.046 $\pm$ 0.002 μM ; IFN- γ stimulated: L-KYN, 29.3 $\pm$ 0.9 μM ; QUIN, 1.58 $\pm$ 0.013 μM .

stimulatory effects of IFN- γ [21]. The present results show that, in THP-1 cells, TNF- α alone slightly increased L-KYN and [$^{13}\text{C}_6$]QUIN production, and slightly enhanced their accumulation in response to IFN- γ . Both IFN- γ and TNF- α expression are increased in brain and systemic tissues in a broad spectrum of inflammatory conditions, and may therefore have regulatory roles in accelerating kynurenine-pathway metabolism. No conversion of [$^{13}\text{C}_6$]anthranilic acid into [$^{13}\text{C}_6$]QUIN was detected in THP-1 cells. By inference, therefore, macrophages contain indoleamine 2,3-dioxygenase, kynurenine 3-hydroxylase, kynureninase and 3-hydroxyanthranilate 3,4-dioxygenase. Increased activities of indoleamine 2,3-dioxygenase, kynurenine 3-hydroxylase, kynureninase and 3-hydroxyanthranilate 3,4-dioxygenase have been demonstrated in a model of brain injury and inflammation [10].

No [$^{13}\text{C}_6$]QUIN was detected in primary cell cultures of rat microglia, the 'resident macrophage' of the brain. This observation supports the notion that brain microglia may have biochemical properties different from those of macrophages [22]. Infiltration of monocytes occurs following infection of the brain by micro-organisms, following brain injury and during auto-

immune processes, and also occurs in systemic tissues during inflammation. Thus, although the brain and certain systemic tissues may not normally be able to convert either L-tryptophan or L-KYN into QUIN, infiltration of monocytes may be an important source of QUIN in both the central nervous system and the periphery. It is also possible that kynurenine-pathway metabolites synthesized in one cell type may enter the blood to be converted into QUIN by other cells, such as those in the liver [4,5,11].

Rat primary astrocytes and human U373MG cells did not synthesize [$^{13}\text{C}_6$]QUIN from L-[$^{13}\text{C}_6$]tryptophan, although they were able to synthesize L-KYN in response to IFN- γ (Table 1). Astrocytes do contain 3-hydroxyanthranilate 3,4-dioxygenase, the enzyme for QUIN formation. An increase in the activity of 3-hydroxyanthranilate 3,4-dioxygenase, however, such as occurs in patients with Huntington's disease [23], does not result in an accumulation of QUIN in brain tissue [2,24], presumably because the concentrations of 3-hydroxyanthranilic acid are extremely low in the brain [25]. Therefore changes in the activity of 3-hydroxyanthranilate 3,4-dioxygenase may only have an impact on QUIN formation if there is an increase in the synthesis of 3-hydroxyanthranilic acid. The presence of activated macrophages, which release 3-hydroxyanthranilic acid [20], would be one circumstance. Direct application of 3-hydroxyanthranilic acid to brain results in marked increases in brain QUIN concentrations [13], which suggests that 3-hydroxyanthranilate 3,4-dioxygenase is not saturated with substrate and is not the rate-limiting enzyme in the synthesis of QUIN by brain tissue. Clearly, changes in the activity of 3-hydroxyanthranilate 3,4-dioxygenase are not a prerequisite for increased QUIN synthesis.

Interestingly, both hepatic cell lines studied (SKHep1 and HepG2) also responded to IFN- γ with increased synthesis of L-KYN, although only SKHep1 cells actually synthesized [$^{13}\text{C}_6$]QUIN. The liver contains an enzyme distinct from indoleamine 2,3-dioxygenase, tryptophan-2,3-dioxygenase, which is not induced by IFN- γ [7,11], and its activity has generally been viewed as the determinant of L-tryptophan catabolism through the kynurenine pathway and QUIN synthesis [26]. The present results (Table 1) suggest that certain liver cells also contain indoleamine 2,3-dioxygenase, and may be a source of kynurenine-pathway metabolites in blood following systemic immune activation. The liver may further contribute to QUIN synthesis by uptake and metabolism of kynurenine-pathway metabolites that are synthesized in extrahepatic tissues, and then released into the blood in response to indoleamine 2,3-dioxygenase induction. The increases in brain kynurenine-pathway metabolites may also represent entry of systemically synthesized compounds [4,10,11]. However, it should be noted that an accumulation of kynurenine-pathway metabolites within the brain occurs independently of systemic involvement in conditions of intracerebral immune activation [3,4,10].

Indoleamine 2,3-dioxygenase has broad substrate specificity for indoles, without stereospecificity, and cleaves the pyrrole ring of substrates, including L-tryptophan, D-tryptophan, 5-hydroxytryptamine and β -carbolines [14]. Compounds such as norharmane inhibit indoleamine 2,3-dioxygenase with relatively low affinity (K_i of 120 μM for rabbit intestine). In the present study, the amounts of both QUIN and L-KYN produced by THP-1 cells were significantly reduced by norharmane in a dose- and time-dependent manner (Figure 1). The effects of norharmane and 6-chloro-DL-tryptophan can be attributed to the inhibition of indoleamine 2,3-dioxygenase activity [14]. 4-Chloro-

3-hydroxyanthranilate is an inhibitor of 3-hydroxyanthranilate 3,4-dioxygenase [12,13]. The present results show that 4-chloro-3-hydroxyanthranilate was a potent inhibitor of [$^{13}\text{C}_6$]QUIN synthesis from L-[$^{13}\text{C}_6$]tryptophan by THP-1 cells, but had no effect on L-kynurenine synthesis. Further studies are required to define the mechanisms by which 6-chloro-DL-tryptophan, 4-chloro-3-hydroxyanthranilate and norharmane attenuate QUIN synthesis.

Collectively, these results suggest that monocytes and certain liver cells may be important cell types that can directly convert L-tryptophan into QUIN following immune stimulation. The exchange of kynurenine-pathway metabolites between cell types and organs, however, could by-pass rate-limiting enzymes and render cell types that would otherwise be unable to synthesize QUIN able to influence QUIN synthesis. THP-1 cells are a useful tool to screen for drugs which can attenuate the formation of QUIN and other neuroactive kynurenine-pathway metabolites in inflammatory conditions.

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