

Different kynurenine pathway enzymes limit quinolinic acid formation by various human cell types

Melvyn P. HEYES^{*1}, Cai Y. CHEN^{*}, Eugene O. MAJOR[†] and Kuniaki SAITO^{*}

^{*}Laboratory of Neurotoxicology, NIMH, 9000 Rockville Pike, Bethesda MD 20892-1262, U.S.A. and [†]Laboratory of Viral and Molecular Pathogenesis, NINDS, 9000 Rockville Pike, Bethesda, MD 20892, U.S.A.

Substantial increases in the tryptophan–kynurenine pathway metabolites, L-kynurenine and the neurotoxin quinolinic acid, occur in human brain, blood and systemic tissues during immune activation. Studies *in vitro* have shown that not all human cells are capable of synthesizing quinolinate. To investigate further the mechanisms that limit L-kynurenine and quinolinate production, the activities of kynurenine pathway enzymes and the ability of different human cells to convert pathway intermediates into quinolinate were compared. Stimulation with interferon γ substantially increased indoleamine 2,3-dioxygenase activity and L-kynurenine production in primary peripheral blood macrophages and fetal brains (astrocytes and neurons), as well as cell lines derived from macrophage/monocytes (THP-1), U373MG astrocytoma, SKHEP1 liver and lung (MRC-9). High activities of kynurenine 3-hydroxylase, kynureninase or 3-hydroxyanthranilate 3,4-dioxygenase were found in interferon- γ -stimulated macrophages, THP-1 cells and SKHEP1 cells, and these cells

made large amounts of quinolinate when supplied with L-tryptophan, L-kynurenine, 3-hydroxykynurenine or 3-hydroxyanthranilate. Quinolinate production by human fetal brain cultures and U373MG cells was restricted by the low activities of kynurenine 3-hydroxylase, kynureninase and 3-hydroxyanthranilate 3,4-dioxygenase, and only small amounts of quinolinate were synthesized when cultures were supplied with L-tryptophan or 3-hydroxyanthranilate. In MRC-9 cells, quinolinate was produced only from 3-hydroxykynurenine and 3-hydroxyanthranilate, consistent with their low kynurenine 3-hydroxylase activity. The results are consistent with the notion that indoleamine 2,3-dioxygenase is an important regulatory enzyme in the production of L-kynurenine and quinolinate. Kynurenine 3-hydroxylase and, in some cells, kynureninase and 3-hydroxyanthranilate 3,4-dioxygenase are important determinants of whether a cell can make quinolinate.

INTRODUCTION

The kynurenine pathway is the major route of L-tryptophan catabolism and an anabolic source of nicotinamide-containing nucleotides (Scheme 1). The liver is a major site of systemic kynurenine pathway metabolism, and substrate flux is predominantly regulated by tryptophan 2,3-dioxygenase [1]. This enzyme has a high substrate specificity for L-tryptophan and is induced by corticosteroids. In extrahepatic tissues, however, the first enzyme of the kynurenine pathway is indoleamine 2,3-dioxygenase (IDO) rather than tryptophan 2,3-dioxygenase. This enzyme has a much broader substrate profile for indoleamine-containing compounds and is induced by interferon γ [2].

In brain, L-tryptophan is metabolized to 5-hydroxytryptamine and other indoleamines rather than via the kynurenine pathway. Consequently, the concentrations of the convulsant, L-kynurenine, and the neurotoxin and *N*-methyl-D-aspartate receptor agonist, quinolinic acid (QUIN), are normally lower in brain than blood and systemic tissues. During immune activation, however, the activity of IDO and the levels of L-kynurenine and QUIN increase substantially [3–7]. If immune activation is systemic, such as in septicemia or after intraperitoneal injections of endotoxin or interferon γ , both brain and systemic tissue IDO activities are increased, and L-kynurenine and QUIN in blood is derived from several systemic extrahepatic organs, while the elevations in brain QUIN reflect entry from the blood as well as *de novo* synthesis [5–7]. If immune activation is within the central nervous system, however, blood L-kynurenine and QUIN levels are unchanged, and QUIN is predominantly synthesized within the brain from L-tryptophan and perhaps other precursors in association with large local induction in IDO activity in microglia,

astrocytes and invading macrophages [7–10]. Microglia and macrophage infiltrates may be particularly important sites of QUIN synthesis within the brain [11,12].

In a previous study of human primary peripheral blood macrophages and fetal brain cells, as well as cell lines derived from macrophage/monocytes, lung and liver, induction of IDO by interferon γ was shown to increase L-kynurenine production [9,11,13]. Notably, however, the ability of these cell types to produce QUIN from L-tryptophan was restricted. It is not known which enzyme(s) are involved in limiting the cell's ability to convert L-kynurenine into QUIN, although there is evidence that kynurenine 3-hydroxylase may be a regulatory step [1,10,14,15]. In the present study, the activities of indoleamine 2,3-dioxygenase, kynurenine 3-hydroxylase, kynureninase and 3-hydroxyanthranilate 3,4-dioxygenase in primary cultures of human peripheral blood macrophages and fetal brain cells, consisting of approx. 40% neurons and 60% astrocytes, and in human cell lines, THP-1 (monocyte/macrophage), U373MG (astrocytoma), SKHEP1 (liver), WIL-NS (B-cell) and MRC-9 (lung), were determined. These cells were either unstimulated or stimulated with interferon γ QUIN production was also quantified when these cells were supplied with increased amounts of kynurenine pathway intermediates to evaluate which are the rate-limiting steps in QUIN synthesis.

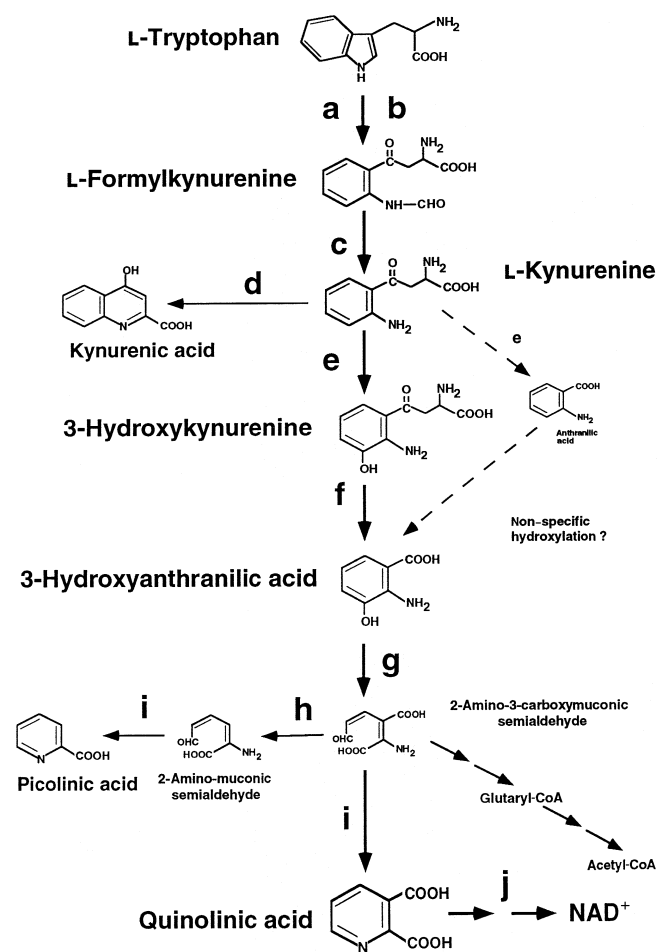
MATERIALS AND METHODS

Chemicals

Quinolinic acid, 1,1,1,3,3,3-hexafluoroisopropanol, L-kynurenine, 3-hydroxy-DL-kynurenine and 3-hydroxyanthranilic acid

Abbreviations used: IDO, indoleamine 2,3-dioxygenase; QUIN, quinolinic acid; TNF- α , tumour necrosis factor α ; LPS, lipopolysaccharide.

¹ To whom correspondence should be addressed.



Scheme 1 Simplified overview of the metabolites and enzymes of the kynurenine pathway

(a) Indoleamine 2,3-dioxygenase, EC 1.13.11.17; (b) tryptophan 2,3-dioxygenase, EC 1.13.11.11; (c) kynurenine formamidase, EC 3.5.1.9; (d) kynurenine aminotransferase (2-oxoglutarate aminotransferase), EC 2.6.1.7; (e) kynurenine 3-hydroxylase, EC 1.14.13.9; (f) kynureninase, EC 3.7.1.3; (g) 3-hydroxyanthranilate 3,4-dioxygenase (3-hydroxyanthranilic acid oxidase), EC 1.13.11.6; (h) picolinic carboxylase, EC 4.1.1.45; (i) non-enzymic cyclization; (j) quinolinic acid phosphoribosyltransferase, EC 2.4.2.19.

were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Trifluoroacetylimidazole was acquired from Pierce Chemical Co. (Rockford, IL, U.S.A.). L-[ring-¹³C₆]Tryptophan was from MSD Isotopes (Montreal, Canada). [³H₂]QUIN and [¹³C₆]anthranilic acid were obtained from Le Research, St. Paul, MN, U.S.A. RPMI 1640, L-glutamine, fetal bovine serum, gentamicin, penicillin and streptomycin were obtained from Biofluids Inc. (Rockville, MD, U.S.A.). Human recombinant interferon γ was obtained from Amgen Biologicals (Thousand Oaks, CA, U.S.A.), and remaining reagents were obtained from Fisher Inc. (Fair Lawn, NJ, U.S.A.).

Culture conditions

Peripheral blood macrophages from human volunteers were obtained from ABI Advanced Biotechnologies Inc. (Columbia, MD, U.S.A.). Macrophages were incubated in a 75 cm² plastic culture flask at a density of 10⁷ cells per flask in 20 ml of tissue culture medium consisting of Dulbecco's modified Eagle's me-

dium (high glucose) with 4 mM L-glutamine, 20% fetal bovine serum and 10% human AB+ serum.

Cultures of human fetal brains [16] were incubated in Eagle's minimal essential medium supplemented with 10% fetal bovine serum and 25 μ g/ml gentamicin. Cultures consisted of a heterogeneous population of cells identified as astrocytes (approx. 65%) and neurons (approx. 25%) by using a monoclonal antibody to either the intermediate filaments of glial fibrillary acidic protein (Lab Systems, Amsterdam, The Netherlands) or neurofilament (Boeringer-Mannheim, Mannheim, Germany). Although no myelin or myelin basic protein was detected in these cultures, a few cells (< 1%) were gal/C-positive and have been referred to as oligodendrocyte progenitor cells. Cells were expanded in culture into 25 cm² flasks and used at the third passage (2.3 \times 10⁶ cells/flask). At this generation period, there is no evidence of microglial cells [17] when tested for Fc receptors and reactivity to macrophage markers, including Mac 1s. Cells were incubated in L-tryptophan-free RPMI 1640 medium containing 50 μ M L-[¹³C₆]tryptophan, supplemented with 10% heat-inactivated fetal bovine serum and 2 mM L-glutamine at 37 °C under humidified air containing 5% CO₂.

THP-1, U373MG, SKHEP1, WIL-NS and MRC-9 cells were obtained from the American Type Culture Collection [18] (Rockville, MD, U.S.A.). THP-1 cells exhibit monocytic characteristics, including phagocytosis and expression of Fc receptors, U373MG cells are an astrocyte cell line, SKHEP1 cells are a liver tumour cell line, WIL-NS cells are a B-cell line and MRC-9 cells were derived from a normal human fetal lung. THP-1 and WIL-NS cells are non-adherent cells and are grown in 75 cm² flasks in 12 ml of RPMI 1640 medium (BioWhittaker, Walkersville, MD, U.S.A.) containing 10% fetal bovine serum and 2 mM L-glutamine. THP-1 cell culture medium also contained 20 μ M 2-mercaptoethanol. Cells were centrifuged at 200 g (1000 rev./min) for 10 min at room temperature. The supernatant was replaced with medium, supplemented with 10% heat-inactivated fetal bovine serum and 2 mM L-glutamine. Cells were transferred to six-well plates [approx. (2–4) \times 10⁵ cells/ml in 3 ml of tissue culture medium]. U373MG, SKHEP1 and MRC-9 cells are adherent cells and are grown in 75 cm² flasks in 12 ml of RPMI 1640 medium containing 10% fetal bovine serum and 2 mM L-glutamine. On the day before study, the cells were washed with 12 ml of RPMI 1640 medium, and 6 ml of trypsin/versene (BioWhittaker) was added. After 5 min, cells detached and 6 ml of fetal bovine serum was added. Cells were centrifuged, resuspended in the Eagle's minimal essential medium and transferred to six-well plates (5 \times 10⁵ cells/ml). On the day of study, the cells were washed with 3 ml of RPMI and incubated in L-tryptophan-free RPMI 1640-containing medium, supplemented with 10% heat-inactivated fetal bovine serum and 2 mM L-glutamine.

Cells were either unstimulated as controls, or stimulated with lipopolysaccharide (LPS), interferon γ or tumour necrosis factor α (TNF- α). Potential QUIN precursors were added to cultures of blood macrophages, THP-1 cells, human fetal brain, U373MG and MRC-9 cells: L-[¹³C₆]tryptophan, L-kynurenine, 3-hydroxy-DL-kynurenine, [¹³C₆]anthranilic acid and 3-hydroxyanthranilic acid. The data presented are from 48 h of incubation, a time when L-kynurenine and [¹³C₆]QUIN concentrations in the medium are approximately maximal.

Biochemical analyses

All samples within any given set of tissues or experiments were run within the same assay with appropriate standard curves [5,6,9,10,14,19].

For enzyme assays, samples were homogenized in 1 ml of 50 mM PBS, pH 7.0, for the IDO, kynureninase and 3-hydroxyanthranilate 3,4-dioxygenase assays, or ice-cold 0.32 M sucrose for the kynurenine 3-hydroxylase assay. The buffer extracts were shaken at 37 °C for 60 min (IDO), 30 min (kynureninase and 3-hydroxyanthranilate 3,4-dioxygenase) or 10 min (kynurenine 3-hydroxylase). After incubation, the reaction was terminated with 0.1 ml of 20% (w/v) trichloroacetic acid or 3.0 M HCl (3-hydroxyanthranilate 3,4-dioxygenase assay). Incubation mixtures were centrifuged at 12000 g for 10 min, and the enzymic products measured. Enzyme activity (V_{max}) was calculated from the difference between product content before (blanks) and after incubation, and expressed as the amount of product formed/unit time per tissue amount (pmol/h per mg of protein).

IDO activity was quantified by measuring the conversion of L-tryptophan into L-kynurenine in a reaction mixture that consisted of 50 μ l of cell homogenate supernatant and 50 μ l of 100 mM potassium phosphate buffer, pH 6.5, containing 50 μ M Methylene Blue, 10 μ g of catalase, 50 mM ascorbate and 0.4 mM L-tryptophan. After the addition of acid, the mixture was incubated at 50 °C for a further 30 min to hydrolyse *N*-formylkynurenine to L-kynurenine. Kynureninase activity was measured as the conversion of L-kynurenine into anthranilic acid. The reaction mixture consisted of 50 μ l of cell homogenate supernatant and 50 μ l of 200 mM Tris/HCl buffer, pH 8.0, containing 100 μ M pyridoxal phosphate and 2.0 mM L-kynurenine. Kynurenine 3-hydroxylase activity was quantified by measuring the conversion of L-kynurenine into 3-hydroxykynurenine. After homogenization in 10 vol. of ice-cold 0.32 M sucrose, cell extracts were centrifuged at 12000 g for 30 min at 4 °C and the pellet washed three times with 0.32 M sucrose. The pellet was resuspended and sonicated in ice-cold 0.14 M KCl/20 mM potassium phosphate buffer, pH 7.0. The reaction mixture consisted of 50 μ l of suspended tissue homogenate, and 50 μ l of 100 mM potassium phosphate buffer, pH 7.5, containing 4 mM MgCl₂, 3 mM glucose 6-phosphate, 0.4 unit of glucose-6-phosphate dehydrogenase, 0.8 mM NADP⁺ and 2.0 mM L-kynurenine. 3-Hydroxyanthranilate 3,4-dioxygenase activity was quantified by measuring the conversion of 3-hydroxyanthranilic acid into QUIN. The reaction mixture consisted of 50 μ l of cell homogenate supernatant and 50 μ l of 100 mM Mes buffer, pH 6.5, containing 10 μ M ascorbate, 6 mM FeSO₄ and 10 μ M 3-hydroxyanthranilic acid. The minimum sensitivities were: IDO, 0.1 nmol/h per mg of protein; kynureninase, 0.05 nmol/h per mg of protein; kynurenine 3-hydroxylase, 0.1 nmol/h per mg of protein; 3-hydroxyanthranilate 3,4-dioxygenase, 0.01 nmol/h per mg of protein.

[¹³C₆]QUIN and QUIN were measured in incubation medium (100 μ l), homogenized cells and QUIN standards (0.6 to 600 pmol in the same volume as the samples) by electron-capture negative chemical ionization (Hewlett-Packard 5988) with gas chromatography, and either [¹⁸O]QUIN or [³H₂]QUIN added as internal standard [11–13,19]. QUIN, [³H₂]QUIN, [¹⁸O]QUIN and [¹³C₆]QUIN were monitored as the molecular ions (*m/z* 467, 470, 471 and 473) respectively. The minimum detectable limit of QUIN was 50 fg injected (300 amol) on column, or a concentration of 0.2 nM in the original sample. To establish the absence (concentrations < 0.2 nM) of [¹³C₆]QUIN, no [¹⁸O]QUIN or [³H₂]QUIN was added to the original sample (300 μ l), and 2 μ l of a 200 μ l heptane extract (50% extraction efficiency) was injected on column. Ions at *m/z* 473 and the specific fragment at *m/z* 322 were monitored. L-Kynurenine was quantified by HPLC and UV light absorbance spectrophotometry [20]. 3-Hydroxykynurenine was quantified by HPLC and electrochemical detection [14,21]. Anthranilic acid was quantified in incubation buffer by HPLC and fluorescence detection [14].

Protein concentrations were measured by a colorimetric assay (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

Statistical analysis

Values presented are means \pm 1 S.E.M. Results were analysed by one-way analysis of variance with the Mann–Whitney test as the *post hoc* test.

RESULTS

Effects of endotoxin, interferon γ and TNF- α

Interferon γ increased the production of L-kynurenine in all cell types examined (Table 1). LPS also increased L-kynurenine accumulation in all cell types, but in the case of U373MG, SKHEP1, WIL-NS and MRC-9 cells, the magnitude of this increase was substantially less than in blood macrophages and THP-1 cells. TNF- α produced smaller increases in L-kynurenine production by all cell types examined. [¹³C₆]QUIN was detected in blood macrophages, THP-1 cells and SKHEP1 cells, but was not detected in U373MG, WIL-NS and MRC-9 cells in response to LPS, interferon- γ or TNF- α (Table 1). The amount of [¹³C₆]QUIN formed relative to the amount of L-kynurenine was highest in blood macrophages compared with THP-1 and SKHEP1 cells.

Enzyme responses

Compared with interferon- γ , LPS was considerably less potent at increasing IDO activity in U373MG, SKHEP1 and MRC-9 cells, and TNF- α was generally less effective across all cell types (Table 1). Interferon- γ was therefore chosen as the stimulant.

Interferon- γ stimulation resulted in large increases in the activity of IDO in all cell types examined, although the magnitude of the response in blood macrophages was the smallest in both relative and absolute terms (Table 2). Kynurenine 3-hydroxylase activity was detected only in unstimulated blood macrophages, THP-1 and SKHEP1 cells. Note the large difference in their absolute activities. No kynurenine 3-hydroxylase activity was detected in human fetal brain cultures, U373MG, WIL-NS or MRC-9 cells. Interferon γ stimulation produced a small increase in kynurenine 3-hydroxylase activity in THP-1 cells only. Kynureninase activity was detected in all cell types except human fetal brain cultures and WIL-NS cells. Interferon γ slightly increased kynureninase activities in MRC-9 and SKHEP1 cells. 3-Hydroxyanthranilate 3,4-dioxygenase activity was detected in all four cell cultures, but was unchanged by interferon γ stimulation.

Precursor conversion into QUIN

The addition of 3-hydroxyanthranilate to the medium was associated with the production of QUIN in all cell types studied except WIL-NS and U373MG cells (Table 3). The amounts of QUIN that accumulated were very different. QUIN accumulations were highest in blood macrophages, THP-1 and SKHEP1 cells and lowest in the human fetal brain cultures. The addition of 3-hydroxykynurenine to the medium was associated with QUIN production in blood macrophages, THP-1 and SKHEP1 cells. No [¹³C₆]QUIN was detected after the addition of [¹³C₆]anthranilic acid to the incubation medium (not shown). The addition of L-kynurenine enhanced QUIN production in unstimulated blood macrophages and SKHEP1 cells, and slightly enhanced QUIN production by THP-1 cells. Interferon γ stimulation of L-kynurenine-treated cells was associated with a marked increase in QUIN production by THP-1 cells, no change in the

Table 1 Effects of endotoxin, interferon- γ (IFN- γ) and TNF- α on L-kynurenine and QUIN production in different cell types

Cells were incubated with 50 μ M L-[13 C $_6$]tryptophan and either with (maximum responsive dose) or without (control) LPS (1 μ g/ml), IFN- γ (100 units/ml) or TNF- α (100 units/ml). L-kynurenine (μ M; labelled and unlabelled) and [13 C $_6$]QUIN (nM) concentrations after 48 h incubation are expressed as means \pm 1 S.E.M. from at least five incubation wells per time. Note: baseline L-kynurenine levels in the serum added to the incubation media (approx. 0.05 μ M) have been subtracted. The minimum detectable concentrations were: QUIN \leq 0.2 nmol/l, L-kynurenine \leq 0.05 μ M. For statistical comparisons, non-detected metabolites were set at the minimum detectable values. * P < 0.01 compared with control (Mann-Whitney test).

Cell/origin	[L-Kynurenine] (μ M)				[QUIN] (nM)			
	Control	LPS	IFN- γ	TNF- α	Control	LPS	IFN- γ	TNF- α
Blood macrophages	1.1 \pm 0.1	15 \pm 1*	20 \pm 1*	4.0 \pm 0.6*	215 \pm 25	4450 \pm 90*	4620 \pm 94*	405 \pm 74*
THP-1 (monocyte)	0.2 \pm 0.1	26 \pm 2*	41 \pm 4*	0.3 \pm 0.1	6 \pm 1	261 \pm 35*	333 \pm 8*	8 \pm 2
U363MG (astrocytoma)	0.3 \pm 0.1	2.1 \pm 0.3*	43 \pm 1*	0.8 \pm 0.1*	\leq 0.2	\leq 0.2	\leq 0.2	\leq 0.2
SKHEP1 (liver)	0.3 \pm 0.1	0.6 \pm 0.1*	38 \pm 1*	0.6 \pm 0.1*	\leq 0.2	10 \pm 2	523 \pm 22*	0.7 \pm 0.3*
WIL-NS (B-lymphocyte)	\leq 0.05	0.4 \pm 0.1*	0.8 \pm 0.1*	0.1 \pm 0.1*	\leq 0.2	\leq 0.2	\leq 0.2	\leq 0.2
MRC-9 (lung)	\leq 0.05	0.5 \pm 0.1*	41 \pm 1*	0.6 \pm 0.1*	\leq 0.2	\leq 0.2	\leq 0.2	\leq 0.2

Table 2 Effects of interferon- γ (IFN- γ) on kynurenine pathway enzyme activities in different cell types

Cells were incubated with 50 μ M L-[13 C $_6$]tryptophan either with or without (control) 100 units/ml IFN- γ . Values are expressed as means \pm 1 S.E.M. from six incubation wells per time. For statistical comparisons, non-detected metabolites were set at the minimum detectable values. * P < 0.01 compared with control (Mann-Whitney test).

Cell type	IDO (pmol/h per mg of protein)		Kynurenine 3-hydroxylase (pmol/h per mg of protein)		Kynureninase (pmol/h per mg of protein)		3-Hydroxyanthranilate 3,4-dioxygenase (pmol/h per mg of protein)	
	Control	IFN- γ	Control	IFN- γ	Control	IFN- γ	Control	IFN- γ
Blood macrophages	651 \pm 134	7537 \pm 864*	3100 \pm 376	2625 \pm 200	949 \pm 133	992 \pm 104	1333 \pm 376	1353 \pm 376
THP-1 (monocyte)	260 \pm 80	(420 \pm 2) \times 10 3*	363 \pm 6	574 \pm 27*	11 \pm 0.2	12 \pm 0.2	1600 \pm 60	1200 \pm 20
Human fetal brain (astrocytes and neurons)	\leq 0.1	(260.5 \pm 2.2) \times 10 3*	\leq 0.1	\leq 0.1	\leq 0.05	\leq 0.05	37.5 \pm 4.1	43.2 \pm 11.0
U373MG (astrocytoma)	170 \pm 20	(369 \pm 4.6) \times 10 3*	\leq 0.1	\leq 0.1	0.6 \pm 0.1	0.5 \pm 0.39	< 0.01	< 0.01
MRC-9 (lung)	120 \pm 20	(343 \pm 4.6) \times 10 3*	\leq 0.1	\leq 0.1	1.6 \pm 0.1	2.1 \pm 0.39	300 \pm 20	300 \pm 20
WIL-NS (B-lymphocyte)	150 \pm 20	580 \pm 90	\leq 0.1	\leq 0.1	\leq 0.05	\leq 0.05	1.2 \pm 0.1	1.1 \pm 0.1
SKHEP1 (liver)	170 \pm 20	(311 \pm 5.2) \times 10 3*	85 \pm 17	69 \pm 14	3.00 \pm 0.25	3.90 \pm 0.25	1180 \pm 120	1060 \pm 88

Table 3 Selective conversion of kynurenine pathway metabolites into QUIN in different cell types

Cells were incubated with 50 μ M L-[13 C $_6$]tryptophan, 50 μ M L-kynurenine, 50 μ M [13 C $_6$]anthranilic acid, 50 μ M 3-hydroxykynurenine or 50 μ M 3-hydroxyanthranilic acid, and either with or without (control) interferon γ (IFN- γ) (100 units/ml). [13 C $_6$]QUIN and QUIN (nM) concentrations after 48 h incubation (minus the background levels as defined in cultures exposed to no QUIN precursors) are expressed as means \pm S.E.M. from six incubation wells per time. * P < 0.01 compared with control (Mann-Whitney test).

Cell type	QUIN concentration (nM)							
	L-Tryptophan		L-Kynurenine		3-Hydroxykynurenine		3-Hydroxyanthranilic acid	
	Control	IFN- γ	Control	IFN- γ	Control	IFN- γ	Control	IFN- γ
Blood macrophages	500 \pm 35	4843 \pm 212*	4760 \pm 347	3073 \pm 113	3244 \pm 80	1599 \pm 390	5210 \pm 158	3467 \pm 90
THP-1 (monocyte)	1 \pm 1	286 \pm 25*	16 \pm 1	229 \pm 5*	1830 \pm 83	1450 \pm 52	2068 \pm 42	2093 \pm 43
Human fetal brain cultures	Not detected	3 \pm 1*	Not detected	Not detected	Not detected	Not detected	1.2 \pm 1.0	1.5 \pm 1.0
U373MG (astrocytoma)	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected
MRC-9 (lung)	Not detected	Not detected	Not detected	Not detected	176 \pm 5	150 \pm 11	129 \pm 6	105 \pm 5
WIL-NS (B-lymphocyte)	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected
SKHEP1 (liver)	3 \pm 1	267 \pm 30*	473 \pm 33	431 \pm 41	1620 \pm 90	1810 \pm 55	2280 \pm 55	1880 \pm 75

amount of QUIN accumulated by SKHEP1 cells and a small decrease in QUIN accumulations in blood macrophages.

DISCUSSION

The large capacity of all cell types examined to produce L-kynurenine from L-tryptophan in response to interferon γ

stimulation (Table 1) is explained by their large increases in IDO activity (Table 2), notably in blood macrophages. IDO induction is clearly an important initiating event in increasing substrate flux through the kynurenine pathway during immune activation, as L-tryptophan is ingested in substantially higher amounts in the diet than other kynurenine pathway metabolites, and because L-tryptophan is plentiful in both blood and tissues. IDO induction,

however, did not predict a cell's ability to synthesize QUIN. Both MRC-9 cells and human fetal brain cultures had low kynurenine 3-hydroxylase activities, and produced no or very little [$^{13}\text{C}_6$]QUIN respectively in response to interferon γ stimulation. Human fetal brain cells were also relatively deficient in kynureninase and 3-hydroxyanthranilate 3,4-dioxygenase. The ability of human fetal brain cells to synthesize minute amounts of QUIN from L-tryptophan as precursor, however, indicates that they contain at least some kynurenine 3-hydroxylase and kynureninase activity that is below the minimum detection limits of the assay. Blood macrophages, THP-1 and SKHEP1 cells produced [$^{13}\text{C}_6$]QUIN, and contained all the kynurenine pathway enzymes in detectable quantities. Blood macrophages produced the largest amounts of [$^{13}\text{C}_6$]QUIN in accordance with the highest activities of kynurenine 3-hydroxylase and kynureninase of the cell types examined.

The precursor-administration studies (Table 3) produced results consistent with the direct enzyme measurement data. Blood macrophages, THP-1 and SKHEP1 cells synthesized QUIN when supplied with 3-hydroxyanthranilate, 3-hydroxykynurenine, L-kynurenine or L-[$^{13}\text{C}_6$]tryptophan, observations consistent with the presence of all four kynurenine pathway enzymes. One difference between blood macrophages and THP-1 cells was that, whereas interferon γ stimulation of THP-1 cells increased kynurenine 3-hydroxylase activity (Table 2) and enhanced the conversion of L-kynurenine into QUIN (Table 3), blood macrophages actually showed a small decrease in kynurenine 3-hydroxylase activity and a diminished conversion of L-kynurenine into QUIN in response to interferon- γ . The higher quantities of QUIN formed by liver SKHEP1 cells compared with THP-1 cells supplied with L-kynurenine, despite SKHEP1 cells having lower kynurenine 3-hydroxylase and kynureninase activities, may be due to the high uptake and transport capacity that liver cells have for L-kynurenine and other amino acids. There may also be differences in the concentration of intracellular cofactors that influence enzyme activities that are not replicated in the *in vitro* assays. In the MRC-9 lung cell line, no QUIN formation was detected when the cells were supplied with either L-tryptophan or L-kynurenine, whereas large amounts of QUIN were formed when the cells were supplied with either 3-hydroxykynurenine or 3-hydroxyanthranilate. These observations are consistent with the undetectable activity of kynurenine 3-hydroxylase but the presence of both kynureninase and 3-hydroxyanthranilate 3,4-dioxygenase in the lung cell line. In no cell studied was there any change in either kynureninase or 3-hydroxyanthranilate 3,4-dioxygenase activity, or change in the amounts of 3-hydroxykynurenine or 3-hydroxyanthranilate conversion into QUIN, in response to interferon γ stimulation. Anthranilic acid was not converted into QUIN, an observation consistent with the report of Bender and McGregor [22] that anthranilic acid is not a significant source of live and urinary nicotinamide-containing nucleotides.

QUIN production may also be influenced by diversion of 2-amino-3-muconic semialdehyde to picolinic acid and the glutaryl-CoA pathway, as well as conversion of QUIN into nicotinamide-containing nucleotides (Scheme 1). These pathways are particularly active in the liver [1]. Further studies are required to evaluate the roles of picolinic carboxylase, catabolism of 2-amino-3-muconic semialdehyde through the glutaryl-CoA pathway and the activity of quinolinic acid phosphoribosyltransferase in influencing QUIN metabolism in the cell types examined. It should also be noted that L-kynurenine and 3-hydroxykynurenine can be converted into other metabolites that cannot be converted into QUIN. For example, THP-1, SKHEP1, MRC-9, SK-N-SH and U373MG cells convert increased amounts of L-kynurenine

into kynureninic acid in response to interferon γ stimulation [23]. In addition, the availability of substrates for conversion into 2-amino-3-muconic semialdehyde may be influenced by diffusion or active transport of substrates out of the cells. Therefore the availability of 2-amino-3-muconic semialdehyde for QUIN formation may be determined by several metabolic alternatives in addition to the activities of the four enzymes measured in the present study.

In brain-localized inflammation, the activities of kynureninase, kynurenine 3-hydroxylase and particularly IDO are increased [9,10,14,15]. In cerebral ischaemic injury in the gerbil, brain 3-hydroxyanthranilate 3,4-dioxygenase activity is also increased [10]. Blood macrophages infiltrate the brain in a broad spectrum of brain-localized inflammatory conditions, including brain and spinal cord injury, microbiological infections and autoimmune diseases, and in association with substantial elevations in brain QUIN levels [4,24]. Because blood macrophages have high activities of all kynurenine pathway enzymes (Table 2), their infiltration into the brain during inflammation probably contributes appreciably to the reported increases in local enzyme activities [9,10,14,15], particularly as IDO, kynurenine 3-hydroxylase, kynureninase and 3-hydroxyanthranilate 3,4-dioxygenase activities in brain tissue (approx. 6.3, 0.75, 13.1 and 24.1 nmol/h per g wet weight: approx. equivalent to 63, 7.5, 131 and 241 pmol/h per mg of protein respectively; see Table 1 of [9]) are much lower than in blood macrophages (Table 2). Microglia are the resident 'macrophage' in brain and are activated during brain inflammation. Human microglia also synthesize large amounts of QUIN when stimulated with interferon γ [12], although with a slightly lower capacity to synthesize QUIN relative to L-kynurenine than blood macrophages [12]. Microglia may also be important sources of kynurenine pathway enzymes and QUIN synthesis in the normal brain, and during conditions where blood macrophages do not enter the brain.

The present results are consistent with the notion that indoleamine 2,3-dioxygenase is an important regulatory enzyme in the production of L-kynurenine and quinolinate in a broad spectrum of cell types. Kynurenine 3-hydroxylase was consistently deficient in cells with a restricted capacity to synthesize QUIN. In some cells, kynureninase and 3-hydroxyanthranilate 3,4-dioxygenase were also important influences on whether a cell type could synthesize QUIN.

We appreciate the assistance of L. Cornio, B. J. Quearry, M. Saito and M. Zito.

REFERENCES

- Bender, D. A. (1982) *Mol. Aspects Med.* **6**, 101–197
- Carlin, J. M., Ozaki, Y., Byrne, G. I., Brown, R. R. and Borden, E. C. (1989) *Experientia* **45**, 535–541
- Takikawa, O., Yoshida, R., Kido, R. and Hayaishi, O. (1986) *J. Biol. Chem.* **261**, 3648–3653
- Heyes, M. P., Saito, K., Crowley, J., Davis, L. E., Demitrak, M. A., Der, M., Dilling, L., Kruesi, M. J. P., Lackner, A., Larsen, S. A., Lee, K., Leonard, H., Markey, S. P., Martin, A., Milstien, S., Mouradian, M. M., Pranzatelli, M. R., Quearry, B. J., Salazar, A., Smith, M., Straus, S. E., Sunderland, T., Swedo, S. and Tourtellotte, W. W. (1992) *Brain* **115**, 1249–1273
- Saito, K., Markey, S. P. and Heyes, M. P. (1992) *Neuroscience* **51**, 25–39
- Saito, K., Crowley, J. S., Markey, S. P. and Heyes, M. P. (1993) *J. Biol. Chem.* **268**, 15496–15503
- Heyes, M. P. and Morrison, P. F. (1997) *J. Neurochem.* **68**, 280–288
- Heyes, M. P., Saito, K., Jacobowitz, D., Takikawa, O., Markey, S. P. and Vickers, J. (1992) *FASEB J.* **6**, 2977–2989
- Heyes, M. P., Saito, K., Major, E. O., Milstien, S., Markey, S. P. and Vickers, J. H. (1993) *Brain* **116**, 1425–1450
- Saito, K., Nowak, Jr., T. S., Markey, S. P. and Heyes, M. P. (1993) *J. Neurochem.* **60**, 180–192
- Heyes, M. P., Saito, K. and Markey, S. P. (1992) *Biochem. J.* **283**, 633–635

- 12 Heyes, M. P., Achim, C., Wiley, C. A., Major, E. O., Saito, K. and Markey, S. P. (1996) *Biochem. J.* **320**, 595–597
- 13 Saito, K., Chen, C. Y., Masana, M., Crowley, J., Markey, S. P. and Heyes, M. P. (1993) *Biochem. J.* **291**, 11–14
- 14 Saito, K., Quearry, B. J., Saito, M., Nowak, T. S., Markey, S. P. and Heyes, M. P. (1993) *Arch. Biochem. Biophys.* **307**, 104–109
- 15 Saito, K., Nowak, T. S., Suyama, K., Quearry, B. J., Saito, M., Crowley, J. S., Markey, S. P. and Heyes, M. P. (1993) *J. Neurochem.* **61**, 2061–2070
- 16 Major, E. O. and Vacante, D. (1989) *J. Neuropathol. Exp. Neurol.* **48**, 425–436
- 17 Elder, G. and Major, E. O. (1988) *Dev. Brain Res.* **42**, 146–150
- 18 Hay, R., Macy, M., Chen, T. R., McClintock, P. and Reid, Y. (1988) American Type Culture Collection Catalog of Cell Lines and Hybridomas. 6th Rockville, MD American Type Culture Collection
- 19 Heyes, M. P. and Markey, S. P. (1988) *Anal. Biochem.* **174**, 349–359
- 20 Holmes, E. W. (1988) *Anal. Biochem.* **172**, 518–525
- 21 Heyes, M. P. and Quearry, B. J. (1988) *J. Chromatogr.* **428**, 340–344
- 22 Bender, D. A. and McGregor, G. M. (1982) *Biochim. Biophys. Acta* **717**, 56–60
- 23 Naritsin, D. B., Saito, K., Markey, S. P. and Heyes, M. P. (1995) *J. Neurochem.* **65**, 2217–2226
- 24 Heyes, M. P., Brew, B. J., Martin, A., Price, R. W., Salazar, A. M., Sidtis, J. J., Yergey, J. A., Mouradian, M. M., Sadler, A. E., Keilp, J., Rubinow, D. and Markey, S. P. (1991) *Ann. Neurol.* **29**, 202–209

Received 27 February 1997/23 April 1997; accepted 6 May 1997